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Effects of modulations of the energetic metabolism on the mortality of cultured cells

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Abstract

Since cells are open systems which exchange material with their surroundings, they can be considered as open systems far from equilibrium and in this way, they follow the principles of thermodynamics of open systems. This approach stresses the fact that cells optimize their use of energy according to their functions. However, with time and/or under environmental challenges, cells can reorganize themselves at other lower levels of energy production and utilization (Toussaint et al. (1991) Mech. Ageing Dev. 61, 45–64). Considered as optimized systems, cells can adapt their behaviours according to the balance between, on one side, their energetic potential and the level of their defence systems, and on the other side, the intensity of the stress. Mainly three types of behaviour can be theoretically predicted. If the stresses are very low, the damages generated are instantaneously repaired and the cellular system remains at its steady state of energy production and utilization. If the stresses are of an intermediary intensity, it is predicted that the cell can leave its steady state of energy production and utilization and find a new one characterized by a lower level of entropy production and a higher level of errors. Third, if the stresses are of a very high intensity which can be cytotoxic, the level of the energetic potential of the cell is directly related to cell survival. We tested the latter prediction in the present work in two ways. First, the level of energy production was lowered by partially uncoupling the mitochondria. Then the effect of stresses under *tert*-butylhydroperoxide or ethanol was investigated in order to look for a synergistic effect on cell death with the mitochondria uncoupling. Secondly, the effect of a modification of the energetic sources during the stress was tested. Besides a protective effect found with specific defence systems, the presence of energetic metabolites such as D-glucose, pyruvate/malate, glutamate/malate, was tested and found to be protective. The effect of a stimulator of the energetic metabolism, naftidrofuryl oxalate, was also investigated and found protective. The experimental data provide good evidence that energetic factors can modulate the resistance of cells to various stresses.

Key words: Thermodynamics; Stress; WI-38 fibroblast; Ethanol; *tert*-Butylhydroperoxide; Energetic metabolism; Naftidrofuryl oxalate

1. Introduction

A general relationship between the effects of stresses and cell death can be obtained when cells are considered as global systems which optimize their energy production in order to maintain their structures and their functions, despite the various encountered stresses. Such a regulation of the energy utilization by the cells is necessary for cells to perform their housekeeping functions but also many other specific roles. First, given its differentiation level, each cell has to perform some particular functions like contraction, mitosis, the neosynthesis of specific proteins, etc. Secondly, cells have to resist continuous damage to the molecules or structures by using part of their free energy produced from their nutrient supply. This is mostly obtained by a turnover of cell components with a continuous synthesis of new molecules. Moreover, cells have sophisticated defence and repair mechanisms for resisting environmental challenges such as the presence of free radicals, or any other stressful situation. This consideration of the cells predicts that

Abbreviations: ATP, adenosine triphosphate; BME+S, Eagle culture medium+fetal bovine serum (10% v/v); BME, Eagle culture medium; m-CCCP, *m*-chlorophenylhydrazone carbonyl cyanide; PBS, phosphate-buffered saline; TBHP, *tert*-butylhydroperoxyde.

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changing the level of the defence systems, such as the antioxidant enzymes and the DNA repair enzymes, is not the only possibility to modulate the efficiency of the cell against stressful conditions. Indeed the various defence systems like the repair or elimination systems for damaged proteins, lipids or DNA require free energy, availability of which is limited, and which is shared between numerous cellular functions. In given circumstances, or with aging, a decrease in energy production could reduce the efficiency of these protective systems and increase the cellular susceptibility to a concomitant stress [1].

Such a global view has been obtained on cells considered as open systems far from equilibrium which follow the principles of thermodynamics of open systems. This approach stresses the fact that cells optimize their use of energy according to their functions as described above [1]. However, with time and/or under environmental challenges, cells could reorganize themselves at other lower levels of energy production and utilization, which has been shown to be theoretically possible [1]. In this respect, under stressful conditions, cells could have various behaviours according to the balance between, on one side, their energetic potential and the level of their defence systems, and on the other side, the intensity of the stress, in concentration and duration. Mainly three types of reaction can be theoretically predicted.

If the stress is low, the damages generated are instantaneously repaired and the cellular system remains at its steady state of energy production and utilization, which is called steady state of entropy production dS_i/dt, with dS_i the entropy produced inside the cellular system. In fact, at the steady state, $dS_i/dt + dS_e/dt = 0$, with dS_e/dt being the entropy released by the cell in its environment [1].

If the stress is of an intermediary intensity, it is predicted that the cell can leave its steady state of energy production and utilization and find a new one characterized by a lower level of entropy production and a higher level of errors [1]. This has been well exemplified in studies using in vitro serial cultivation of human fibroblasts as a model of aging. With aging, normal human fibroblasts progress through seven morphotypes with defined biochemical and morphological variations [2,3]. Each of these morphotypes is considered as a different steady state of entropy production. The effect of different types of stresses at a non-lethal intensity like UV light, mitomycin C, strong electromagnetic fields, ethanol or *tert*-butylhydroperoxide, on the human fibroblasts in vitro was to speed up the evolution of the transition from one cell morphotype to the other [2,4–6]. According to the theory, it was also demonstrated that a decrease in the energetic potential of the cell even increased the rate of the accelerated passage [7].

Third, if the stress is of a high intensity, it will be toxic for the cell but, from the theoretical model explained above, the level of the energetic potential of the cell will directly affect the cell resistance to such stressful situations.

In this work, we wanted to test the latter prediction by looking at the role of the energetic metabolism on cell death, using cultivated human fibroblasts as a model. We chose arbitrarily two different kinds of stresses. One is related to the oxygen-derived free radicals and is the incubation of the cells with *tert*-butylhydroperoxide. The second one is the presence of ethanol. Cells have been incubated with one of these two molecules. The influence of these stresses on cell mortality has been registered under various conditions which modulate the energetic metabolism.

2. Materials and methods

2.1. Cell culture

Human diploid WI-38 fibroblasts were purchased from the American Type Culture Collection (USA) and serially cultivated in 75 cm^2 flasks (Cel Cult, UK) as described by Hayflick [8] in BME medium (Flow Laboratories, UK) suppplemented with 10% fetal calf serum (BME + S) (Flow).

2.2. Effect of partial mitochondria uncoupling on cell survival

WI-38 fibroblasts were subcultivated at a density of 700 cells/cm^2 in squared Petri dishes (6 cm diameter, Falcon, USA) and incubated for 24 h in normal conditions. Then BME + S was replaced by BME + S containing different concentrations of cyanide carbonyl *m*-chlorophenylhydrazone (m-CCCP) (Sigma Chemicals, USA), a phenylhydrazone which is a weak acid and can dissipate the electron gradient of the inner mitochondrial membrane (for a review, see [9]). Cells attached to the flask were counted individually every day and considered as surviving cells. This method of cell counting was assessed by Michiels et al. [10] using the dye exclusion test of viability with orange acridine-ethidium bromide [11]. Results were expressed as percentages of surviving cells: the number of cells counted every day were divided by the corresponding number of cells present at day 0 before the incubation under m-CCCP. For each m-CCCP concentration, the results are given as means of four countings ± S.D.

2.3. Effect of partial mitochondria uncoupling on cell survival in stressful conditions

WI-38 fibroblasts were cultivated at a density of 700 cells/cm^2 in squared Petri dishes (6 cm diameter,

Falcon) and incubated for 24 h in normal conditions. Then BME + S was replaced by BME + S containing different concentrations of m-CCCP. After 1/2 h under m-CCCP, fibroblasts were exposed for various incubation times to 10^{-4} M *tert*-butylhydroperoxide (TBHP) or ethanol 4% (v:v) diluted in phosphate-buffered saline (PBS) (pH 7.4) 10 mM, NaCl 0.15 M containing the same m-CCCP concentration and at 37°C, or under various TBHP concentrations ranging from $2.5 \cdot 10^{-5}$ M to 10^{-4} M for 1 h. Then the cells were rinsed twice with BME and were incubated in BME + S containing the same m-CCCP concentration than during the stress. Cells attached to the flask were counted individually at day 1 following the stress and considered as surviving cells; the results are expressed as percentages of surviving cells as explained above. A synergistic effect was calculated at day 1 after the stress by subtracting, from the percentage of cell loss observed in the presence of both molecules (m-CCCP + TBHP in one case, and m-CCCP + ethanol in the other case), the percentages of cell loss obtained for each one separated. In each case, the results are given as means of four countings ± S.D.

2.4. Effect of the medium composition on cell mortality

WI-38 fibroblasts were subcultivated in multi-dishes (24 wells, Cel Cult). When confluence was reached, the stress was performed for 1 h in the following way: the culture medium was removed and replaced either by PBS alone, PBS + the amino acids present in BME + essential amino acids (Gibco, UK), PBS + the vitamins (Gibco) present in BME, PBS + 5 mM D-glucose (Merck, Germany), PBS with 10% fetal calf serum, BME, or BME + S, containing TBHP at various concentrations ranging from $5 \cdot 10^{-5}$ to $5 \cdot 10^{-3}$ M for 1 h. Then cells were rinsed twice with BME alone and were incubated in BME + S for 2 days. Similar stresses were performed in PBS containing 4 mg/ml bovine albumin (Sigma) or after a pre-incubation of cells with vitamin E (Sigma) at various concentrations ranging from $5 \cdot 10^{-5}$ to $5 \cdot 10^{-4}$ M. Vitamin E was first dissolved in pure ethanol (Merck) then diluted in BME + S for the final concentrations so that the maximum ethanol concentration was always under 0.1% (v/v) in the presence of cells. At day 2 following the stress, cells were rinsed twice with PBS. 0.6 ml of 0.5 N NaOH was added in each well containing the surviving cells. The amount of proteins in each well was then measured according to the classical method described by Lowry et al. [12]. For each medium, results are expressed as percentages of proteins present in control wells without TBHP at day 2 considered as 100% and are given as means of four countings ± S.D.

2.5. Effect of glucose, pyruvate-malate, glutamate-malate or naftidrofuryl on cell mortality

WI-38 fibroblasts were subcultivated in multi-dishes (24 wells, Cel Cult). When confluence was reached, the stress was performed in the following way: the culture medium was removed and replaced by TBHP at various concentrations ranging from $5 \cdot 10^{-5}$ to $5 \cdot 10^{-3}$ M for 1 h diluted either in PBS alone, PBS + 5 mM D-glucose (Merck), PBS + 5 mM L-glucose (Sigma), PBS + 5 mM pyruvate + 1 mM malate (Sigma), PBS + 5 mM phosphoenolpyruvate (Sigma) + 1 mM malate, PBS + 5 mM glutamate (Sigma) + 1 mM malate, PBS + naftidrofuryl oxalate (Praxilene®, Lipha, France) which is described in literature as a stimulator of the energetic metabolism [13,14]. Cells were then rinsed twice with BME alone and were incubated in BME + S for 2 days. At day 2 following the stress, cells were rinsed twice with PBS and the protein content of each well measured as described above.

2.6. Measurements of ATP concentrations

WI-38 fibroblasts were subcultivated in multi-dishes (24 wells, Cel Cult). When confluence was reached, which represents about 100 000 cells in each well, a stress was performed in the following way: the culture medium was removed and replaced for 1 h by TBHP $5 \cdot 10^{-5}$ M diluted in PBS alone, PBS + 5 mM pyruvate + 1 mM malate, PBS + 5 mM D-glucose, PBS + 5 mM glutamate + 1 mM malate or PBS + 10^{-5} M naftidrofuryl oxalate.

ATP measurements were performed before the stress under TBHP, at different moments during the stress and just after the stress. After rinsing twice with PBS, cells were covered by 0.5 ml of 'Somatic Cell Releasing Reagent' (Sigma) at 4°C and 50 μl of this medium containing the ATP of cell lysis were collected and stored at −70°C for ATP measurements. The remaining releasing reagent was removed and used for protein measurements. The amount of proteins still attached to the flask after removing the releasing reagent was also determined by the classical method described by Lowry et al. [12]. The amount of ATP was determined using a bioluminescence technique: the bioluminescent somatic cell assay kit (Sigma). The luminescence was measured by a luminometer (Lumac Biocounter 2010, Switzerland) with transforms the received light into electric output. The quenching effect of the cellular components was estimated by comparing the standard curve obtained in the presence or not of cell lysate. It was found to be 12.4% in these experimental conditions. The detection limit was found to be 1 pmol ATP/μg cellular protein and linearity of the

relationship between ATP amount and relative light units exists up to 25 pmol ATP/μg cellular protein. The amount of ATP present in each cell lysate was measured and expressed in pmol ATP/μg cellular protein. The results are given as percentages of the ATP compared to the control after an incubation of 1 h in PBS alone and are presented as means of four countings $\pm$S.D. The ATP content of the cells after 1 h incubation in PBS was very close to the initial content and represented 93.2% $\pm$ 6.9% of the non-incubated cells.

2.7. Incorporation of tritiated thymidine into DNA

WI-38 fibroblasts were subcultivated in Petri dishes (3.5 cm diameter, Falcon). One day later, cells were incubated under TBHP $5 \cdot 10^{-5}$ M as explained above. Between day 1 and day 2 after the stress, cells were incubated with BME + S + [^{3}H]thymidine (specific activity 2 Ci/mmol, NEN, UK). After the incubation, cells were fixed and autoradiography was performed as classically described by Cristofalo and Sharf [15]. Results are given as the percentages of labelled

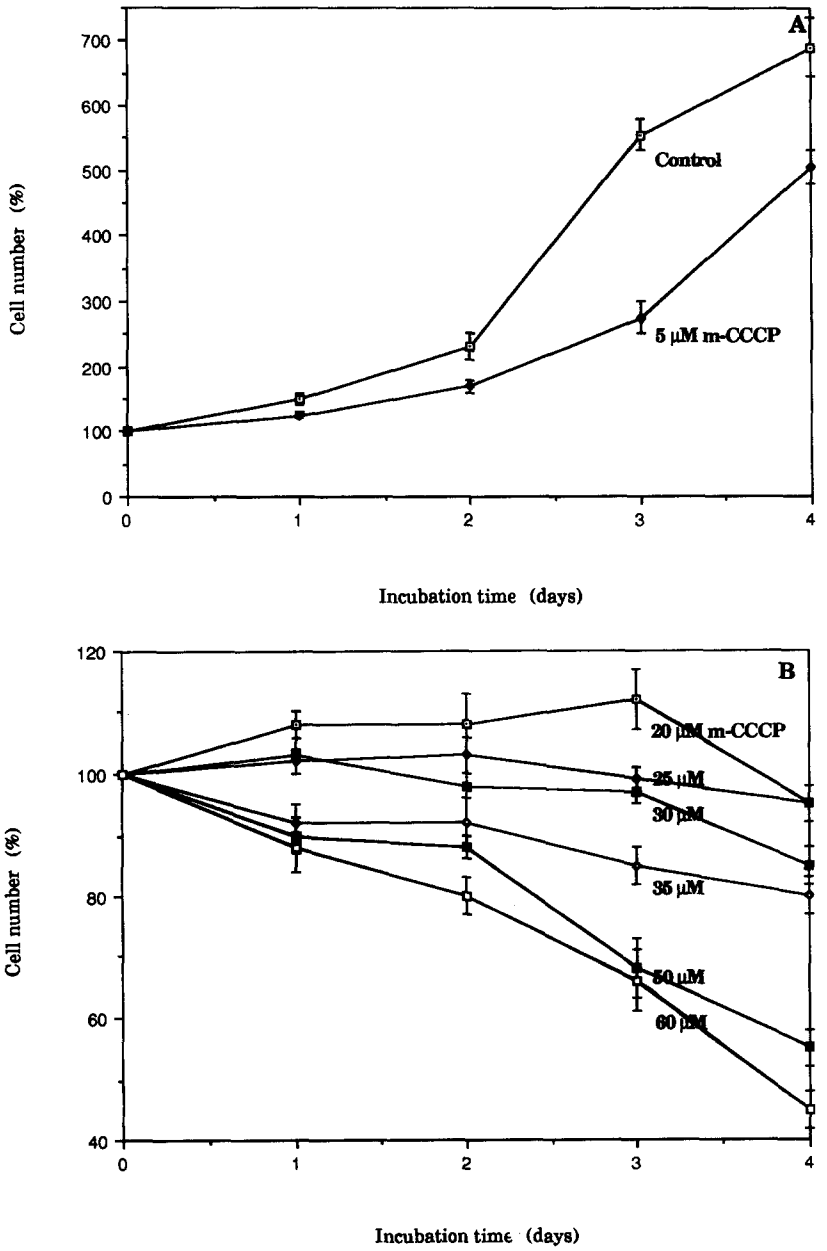

Fig. 1. Effect of m-CCCP on cell survival. WI-38 fibroblasts subcultivated at a density of 700 cells/cm^2 were incubated for several days in BME + S containing m-CCCP at various concentrations. The numbers of cells were determinated every day by cell countings. The results were calculated as the numbers of cells counted every day for each m-CCCP concentration and are expressed in percentages of the numbers of cells present before the incubation ($\pm$S.D. on four experiments) versus the incubation time (days). (A) Control (⊡ ——— ⊡), 5 μM m-CCCP (◆). (B) 20 μM (⊡ ——— ⊡), 25 μM (◆), 30 μM (■ ——— ■), 35 μM (◇), 50 μM (■) and 60 μM (□) m-CCCP.

nuclei/total nuclei and are the mean values of two countings of 500 cells/flask.

3. Results

3.1. Effect of partial mitochondria uncoupling on cell survival

WI-38 fibroblasts from between passages 28 and 35 were cultivated at low density with increasing amounts of m-CCCP and the cell counted every day for 4 days. Results presented in Fig. 1 showed a decrease of the proliferation rate at 5 μM m-CCCP (Fig. 1A). At 20 μM m-CCCP, cell death occurs only after 4 days of incubation. At 35, 50 and 60 μM, cell death is observed within the first day of incubation (Fig. 1B). These concentrations leading to cell death are largely above the concentrations leading to mitochondria uncoupling when m-CCCP is used on isolated mitochondria. This is explained by the fact that m-CCCP strongly binds to albumin [16]. It was shown by Corbisier and Remacle [17] that old fibroblasts are more sensitive to uncoupling than young cells. This increase in sensitivity has been attributed to a decrease in the energetic potential of mitochondria. Other factors also affect the ATP production with aging, since Goldstein et al. [18] showed that, in old fibroblasts, there is an increase in glycolysis and a decrease in the proportion of ATP produced by mitochondria. A concentration of 25 μM m-CCCP was chosen for the following experiments since it is not too detrimental for cell survival.

3.2. Effect of partial mitochondria uncoupling on cell survival in stressful conditions

In a first set of experiments, WI-38 fibroblasts were stressed under 10^{-4} M TBHP diluted in PBS for various periods of time ranging between 20 and 60 min in the presence, or not, of 25 μM m-CCCP. Given PBS does not contain albumin, which binds to m-CCCP [16], it can be presumed that the uncoupling effect of the same m-CCCP concentration is higher in PBS than in culture medium supplemented with 10% fetal calf serum (BME + S). 25 μM m-CCCP diluted in BME + S was also supplied to the cells after the stress. One day after the stress, the cell number was estimated and a synergistic effect of both molecules was calculated. The synergistic effect was calculated based on the cell number 24 h after the stress. For example for a 60 min stress under 10^{-4} M TBHP + 25 μM m-CCCP, a 55% decrease in cell number was observed compared to the number of cells present just before the stress, while only a 35% decrease was observed with 10^{-4} M TBHP alone and a −3% decrease with m-CCCP alone (in fact, a 3% increase). The synergistic effect calculated is

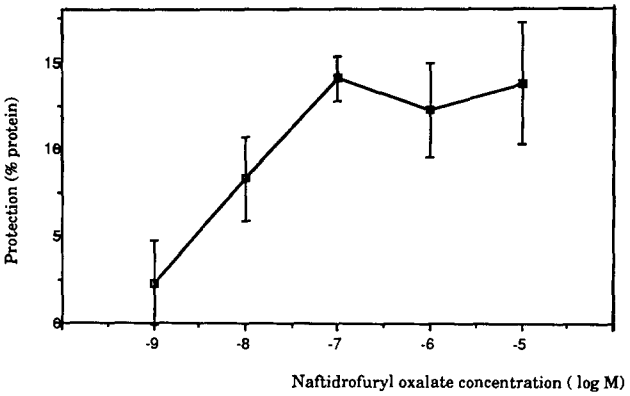

Fig. 2. Effect of various naftidrofuryl oxalate concentrations (log M) on the survival of cells stressed under TBHP. The stresses were performed with $5 \cdot 10^{-5}$ M TBHP on confluent WI-38 fibroblasts in PBS for 1 h, then BME + S was supplied after the stress and the results are expressed in percentages of protection (±S.D. on four experiments) calculated as the increase of protein (in percentage of control) compared to the stress performed without naftidrofuryl.

Table 1
Estimation of the synergistic effect of m-CCCP and TBHP on cytotoxicity

Stresses under TBHP				Stresses under ethanol	
A 10^{-4}M TBHP for various incubation times (min)		**B** TBHP for 1 h at various concentrations (M)		**C** 4% ethanol (v/v) for various incubation times (min)	
Incubation time (min)	Synergistic effect (%)	Concentration (M)	Synergistic effect (%)	Incubation time (min)	Synergistic effect (%)
20	25 ± 3	10^{-4}	16 ± 5	90	16 ± 3
40	26 ± 4	$5 \cdot 10^{-5}$	37 ± 5	105	16 ± 3
60	23 ± 5.5	$2.5 \cdot 10^{-5}$	16 ± 4	120	22 ± 2.5

WI-38 fibroblasts subcultivated at a density of 700 cells/cm^2 were incubated in PBS in the presence of 25 μM m-CCCP or 10^{-4} M TBHP alone or together for various times (**A**). Similar experiments were performed at various TBHP concentrations (M) with or without m-CCCP for 1 h stresses (**B**), or by replacing TBHP by ethanol 4% (v/v) for 1 h stresses (**C**). After rinsing, BME + S was supplied to the cells for 24 h. Cell numbers were then determined by cell countings and expressed in percentages of the cell numbers present before the incubation. A synergistic effect (±S.D. on four experiments) was calculated in these different conditions as follows: the percentage of cell loss observed 24 h after an incubation under TBHP (or ethanol) alone and 24 h after an incubation under m-CCCP alone were subtracted from the percentage of cell loss observed 24 h after an incubation under TBHP (or ethanol) + m-CCCP. An example of calculation is given in the text.

Table 2
Effect of the medium composition on the resistance of cells to TBHP

TBHP concentration (M)	(A) PBS	(B) PBS + aa	(C) PBS + Vit	(D) PBS + D-glucose	(E) PBS + S	(F) BME	(G) BME + S
0	100 ± 3	100 ± 3	100 ± 3	100 ± 3	100 ± 1	100 ± 1	100 ± 2.5
$5 \cdot 10^{-5}$	17 ± 1	16 ± 1	20 ± 2	57 ± 4 *	85 ± 6 *	89 ± 2 *	85 ± 1 *
10^{-4}	16 ± 2	15 ± 2	22 ± 4.5	36 ± 3.5 *	64 ± 3 *	84 ± 1 *	77 ± 1 *
$5 \cdot 10^{-4}$	12.5 ± 2.5	9 ± 1	8 ± 1	39.5 ± 4 *	32 ± 2 *	80 ± 1 *	71 ± 4 *
10^{-3}	15 ± 1.5	9 ± 2	8 ± 2	16 ± 2	32 ± 1.5 *	81 ± 2.5 *	70.5 ± 4 *
$5 \cdot 10^{-3}$	16 ± 3	9 ± 1	12 ± 0.5	18 ± 3	19 ± 2	79 ± 3 *	70 ± 2 *

Cells were incubated for 1 h under various TBHP concentrations from $5 \cdot 10^{-5}$ M to $5 \cdot 10^{-3}$ M diluted in different media. PBS alone (**A**), PBS + amino acids (essential + non-essential) (**B**), PBS + vitamins (**C**), PBS + 5 mM D-glucose (**D**), PBS + S (**E**), BME + S (**F**), BME (**G**). The stresses were performed on confluent WI-38 fibroblasts, then BME + S was supplied after rinsing. The amounts of cellular proteins were determined 2 days after the stress. The results were expressed in percentages of the amount of cellular proteins present in the control (35.5 ± 1.0 μg protein/well) for each medium considered at day 2 after the stress (± S.D. on four experiments). The results of a Student's *t*-test comparing the results presented in column B, C, D, E, F, G to those obtained with PBS alone presented in column A are also presented (* $P < 0.05$).

23% and is the result of 55% (cell loss with TBHP + m-CCCP) − 35% (cell loss with TBHP) − (−3)% (cell loss with m-CCCP). The synergistic effect of 10^{-4} M TBHP + m-CCCP was found to be around 25% for the three stressing times (Table 1A).

Secondly, WI-38 fibroblasts were stressed for 60 min under various TBHP concentrations ranging from $2.5 \cdot 10^{-5}$ M to 10^{-4} M in the presence, or not, of 25 μM m-CCCP. The cell number was estimated in each experiment and the synergistic effect of both molecules on cell mortality was calculated and presented in Table 1B. For each concentration, a synergistic effect was obtained ranging from 18% after a stress under $2.5 \cdot 10^{-5}$ M and 10^{-4} M TBHP M to 37% after a stress under $5 \cdot 10^{-5}$ M TBHP.

Third, a possible synergy between a stress in 4% ethanol and uncoupling was tested in the same way. Results in Table 1C show a synergistic effect ranging from 16% to 22%.

In all these experiments, a significant ($P < 0.05$) synergistic effect on cellular mortality between ethanol or TBHP was obtained with partially uncoupled mitochondria.

3.3. Effect of the medium composition on cell mortality

The thermodynamic theory predicts that cells confronted to stressful conditions increase their energy consumption and therefore either use their storages or increase their intakes of energetic substrates. A first approach to test that cell resistance to stressful conditions depends on the availability of energetic substrates was undertaken by changing the medium in which the stress under TBHP was performed. We used PBS medium containing either amino acids, or vitamins, or glucose or fetal calf serum, and the cell survival was estimated 2 days after the stress under various concentrations of TBHP.

The results presented in Table 2 show that the highest cellular resistance to stresses was obtained when the stress was performed in BME + S (Table 2G). The lowest resistance was found when the stress

Table 3
Effects of albumin, L-glucose, pyruvate-malate, pyruvate, phosphoenolpyruvate, glutamate-malate on cells stressed under TBHP

TBHP concentration (M)	(A) PBS + alb	(B) PBS + L-gluc	(C) PBS + pyr + mal	(D) PBS + pyr	(E) PBS + PEP	(F) PBS + glu + mal	(G) PBS alone
0	100 ± 2	100 ± 4	100 ± 2.5	100 ± 7	100 ± 4	100 ± 3	100 ± 2
$5 \cdot 10^{-5}$	58 ± 4.5 *	19 ± 3	79 ± 3.5 *	74 ± 10 *	20 ± 3	41 ± 2.5 *	17 ± 1
10^{-4}	42 ± 2.5 *	7.5 ± 3	54 ± 3 *	59 ± 5 *	15 ± 3	25 ± 0.5 *	16 ± 2
$5 \cdot 10^{-4}$	19 ± 2.5 *	8 ± 1	40 ± 1.5 *	39.5 ± 1.5 *	16 ± 4	16.5 ± 1 *	12.5 ± 2.5
10^{-3}	18.5 ± 2	11 ± 2.5	35.5 ± 2 *	32 ± 3 *	15 ± 5	15 ± 1.5	15 ± 1.5
$5 \cdot 10^{-3}$	12 ± 3	14 ± 1	20 ± 1	18 ± 5	12 ± 2.5	16 ± 2	16 ± 3

Cells were incubated for 1 h under various TBHP concentrations from $5 \cdot 10^{-5}$ M to $5 \cdot 10^{-3}$ M diluted in different media: either PBS + albumin (4 mg/ml) (**A**), PBS + L-glucose (5 mM) (**B**), PBS + pyruvate (5 mM) + malate (1 mM) (**C**), PBS + pyruvate (5 mM) (**D**), PBS + phosphoenolpyruvate (5 mM) + malate (1 mM) (**E**), PBS + glutamate (5 mM) + malate (1 mM) (**F**) or PBS alone (**G**). The stresses were performed on confluent WI-38 fibroblasts, then BME + S was supplied after rinsing. The amounts of cellular proteins were determined 2 days after the stress. The results were expressed in percentages of the amount of cellular proteins present in the control (37.1 ± 0.7 μg protein/well) for each medium considered at day 2 after the stress (± S.D. on four experiments). The results of a Student's *t*-test comparing the results presented in columns A, B, C, D, E, F to those obtained with PBS alone presented in column G are also presented (* $P < 0.05$).

was performed either in PBS, PBS + amino acids or PBS + vitamins (Table 2A, B and C). Serum diluted in PBS afforded an important protection except at the highest TBHP concentration (Table 2E). D-Glucose diluted in PBS also afforded some protection against $5 \cdot 10^{-5}$ M, 10^{-4} M and $5 \cdot 10^{-4}$ M TBHP (Table 2D). BME gave important protection in all TBHP concentrations tested (Table 2F). The controls obtained 2 days after an incubation in PBS or BME medium without TBHP were very close to each other: 35.5 ± 2.5 μg proteins/well. Incubation for 1 h in PBS alone did not have any consequence on cell growth if cells were taken before passage 35. For older cells, a lack of serum for 1 h led to a lower amount of proteins 2 days after the stress (data not shown). Similar results were obtained when the stressing molecule, here TBHP, was replaced by ethanol from 4% to 7% (v/v) for 2 h incubation (data not shown).

The protection exerted by D-glucose suggests that ATP regeneration during the stress is an important factor in cell resistance to TBHP. Serum protection is more difficult to analyse, since it contains antioxidants such as vitamin E or free radical protectors such as proteins [19]. We first looked for a possible role of proteins and tested various TBHP concentrations on cells in a medium containing albumin at 4 mg/ml. This concentration is the protein concentration present in the medium containing 10% serum used here above. Table 3A shows that albumin afforded some protection, especially at the low TBHP concentrations, when compared with the results obtained with PBS alone. Other factors than albumin present in the culture medium and in the serum could increase the protective effects against TBHP. For instance, vitamin E could be one of these factors. To search for a protective role of vitamin E in protecting the cells against TBHP toxicity in this model, cells were first pre-incubated for 24 h with increasing concentrations of vitamin E ranging from $5 \cdot 10^{-5}$ M to $5 \cdot 10^{-4}$ M. Thereafter, the cells were stressed under $5 \cdot 10^{-5}$ M TBHP for 1 h. The amount of proteins were determined 2 days after the stress. The results were expressed as percentages of the amount of cellular proteins present in the control at day 2 after the stress ($\pm$S.D. on four experiments), as previously. The percentages of proteins obtained without vitamin E were then subtracted from the percentages obtained with vitamin E. Respectively, $29 \pm 5\%$, $33 \pm 1.5\%$ and $40 \pm 3\%$ increases in the percentages of proteins were observed if the stress was performed in the presence of $5 \cdot 10^{-5}$ M, 10^{-4} M or $5 \cdot 10^{-4}$ M vitamin E. These amounts of proteins were all significantly higher ($P < 0.05$) than for the cells treated with TBHP alone, since vitamin E hampers the propagation of lipidic peroxidation from the peroxy radicals [20,21], which are generated by TBHP [22]. Similarly, the protection exerted by the serum observed in Table 2 could have been due to an effect on free radical production and is perhaps not related to any energetic effect.

3.4. Effect of glucose, pyruvate-malate, phosphoenolpyruvate-malate, glutamate-malate and naftidrofuryl oxalate on cell mortality

Since D-glucose was found to reduce the mortality of cells under stress (Table 2), we investigated the effect of molecules which can be used for cell catabolism in order to increase the production of energy. We looked for their influence on the resistance to stresses. First, a control was achieved to assess that the protection afforded by glucose was specific to D-glucose. For that purpose, D-glucose was replaced by L-glucose during stresses under various concentrations in TBHP diluted in PBS and the experiments were performed as previously. The results presented in Table 3B show that no protection could be obtained with L-glucose. The metabolism of glucose is complex and the observed protection could be linked to the ATP turnover, but also to the possibility to regenerate NADPH in the hexose monophosphate shunt, which is necessary for glutathione reductase which in turn regenerates glutathione necessary for the reduction of TBHP by the glutathione peroxidase activity [23,24]. To test for a direct effect on energy metabolism, other metabolites were tested: pyruvate/malate, glutamate/malate and a stimulator of the energetic metabolism: naftidrofuryl.

The presence of pyruvate 5 mM/malate 1 mM diluted in PBS gave an important protection against all TBHP concentrations tested (Table 3C). Pyruvate 5 mM alone afforded a similar protective effect (Table 3D), which can be attributed to the fact that pyruvate like malate can be a source of intermediaries of the Krebs cycle. Also it is possible that changes in the NAD/NADH via the activity of lactate dehydrogenase can affect the mitochondrial and cytosolic metabolism. In starved tumour cells, pyruvate stimulates both compartments, but particularly glycolysis [25]. However, in the case of normal cells, this effect on glycolysis could be less important than in tumour cells, since the mitochondrial oxidative metabolism is higher (for a review, see [26]). When pyruvate 5 mM was replaced by phosphoenolpyruvate 5 mM which cannot enter the cells, no protection was found (Table 3E). Glutamate 5 mM + malate 1 mM diluted in the medium also afforded an important protection against the lowest TBHP concentrations tested (Table 3F).

If glucose was added to glutamate/malate or pyruvate/malate, there was no increase in protection ($3.7 \pm 4.2\%$) (data not shown). This is another argument in favour of a direct energetic role of these molecules besides the fact that they can, however, be used as

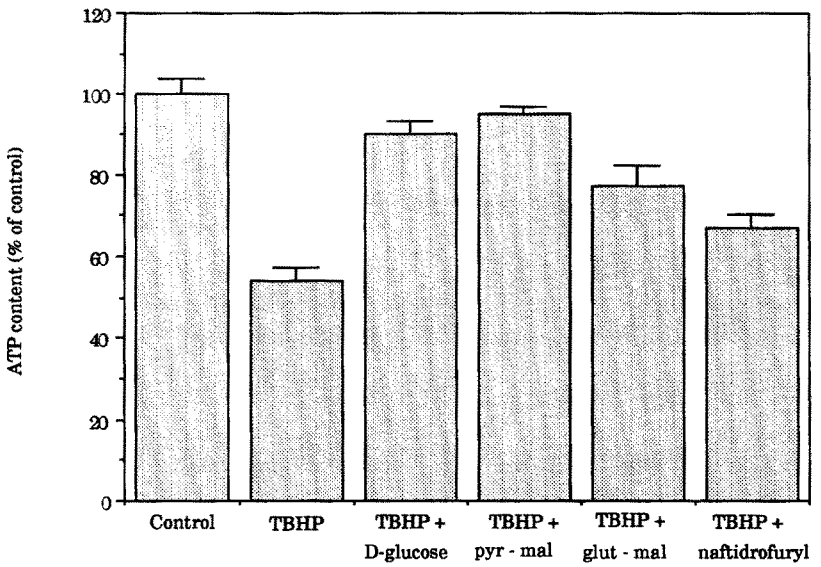

Fig. 3. Effect of either pyruvate + malate, D-glucose, glutamate + malate or naftidrofuryl on the ATP content after a stress under TBHP. WI-38 fibroblasts were stressed for 1 h under $5 \cdot 10^{-5}$ M TBHP diluted in PBS alone, PBS + 5 mM pyruvate + 1 mM malate, PBS + 5 mM D-glucose, PBS + 5 mM glutamate + 1 mM malate or PBS + 10^{-5} M naftidrofuryl oxalate. ATP measurements were performed before the stress under TBHP and just after the stress and are expressed per μg protein. The results are given as percentages of the ATP content compared to the control (2.13 ± 0.11 pmol ATP/μg protein) incubated in PBS alone and are presented as means of four experiments $\pm$S.D. ATP content measured after the incubation in PBS alone was 93.2% $\pm$ 6.9% of the initial value.

substrates for gluconeogenesis and thus increase the activity of the pentose phosphate shunt.

A more pharmacological approach to the protection was undertaken with naftidrofuryl oxalate which was added at various concentrations in PBS containing $5 \cdot 10^{-5}$ M TBHP. The amount of proteins was esti-

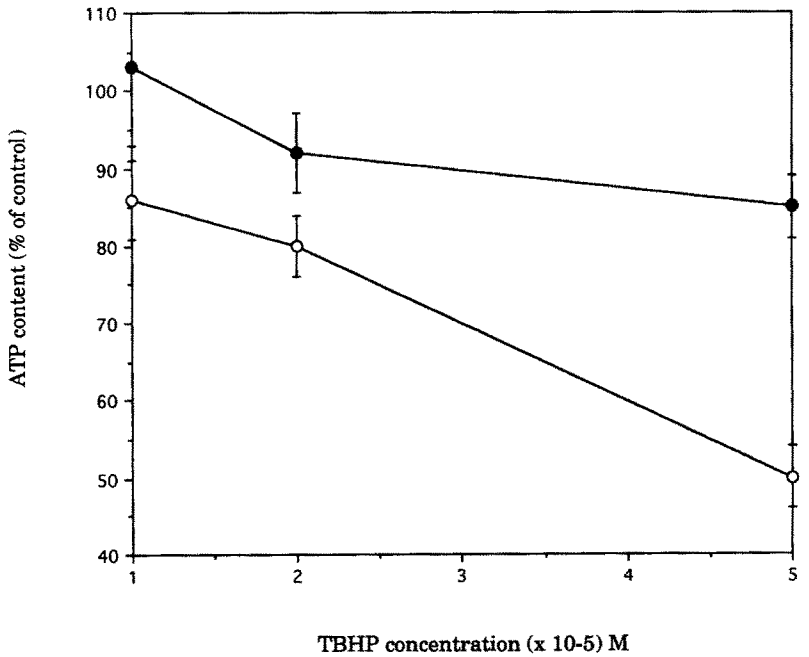

Fig. 4. Variation of the ATP cellular content after stresses under various TBHP concentrations. WI-38 fibroblasts were stressed for 1 h under various TBHP concentrations (M) diluted in PBS alone (○) or PBS + 5 mM pyruvate + 1 mM malate (●). ATP measurements were performed before the stress under TBHP and just after the stress and expressed per μg protein. The results are given as percentages of the ATP content compared to the control (2.20 ± 0.22 pmol ATP/μg protein) incubated for 1 h in PBS alone and are presented as means of four experiments $\pm$ S.D.

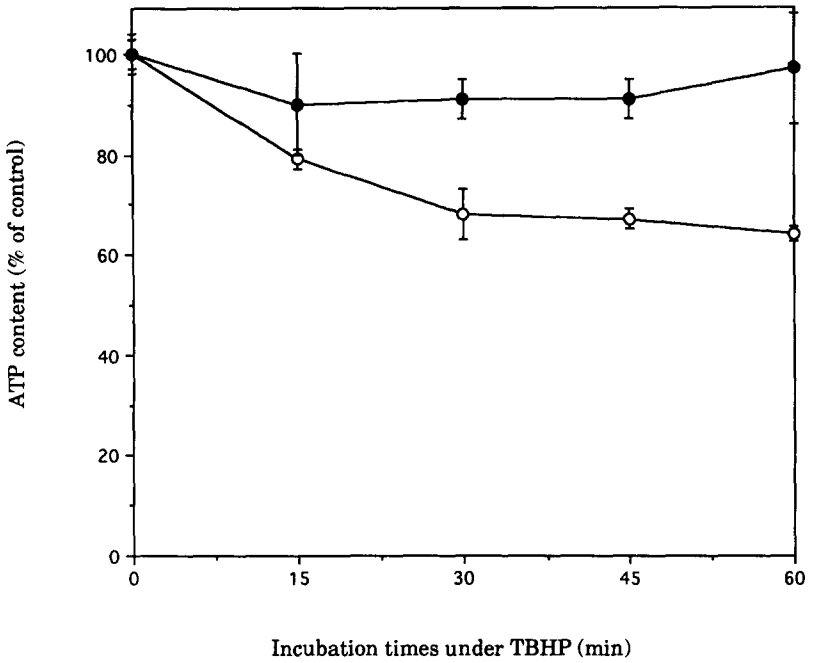

Fig. 5. Evolution of the ATP cellular content during a stress under TBHP. WI-38 fibroblasts were stressed for 1 h under $5 \cdot 10^{-5}$ M TBHP diluted in PBS alone (columns) or PBS + 5 mM D-glucose (line). ATP measurements were performed before the stress under TBHP, at different moments during the stress and just after the stress and are expressed per μg protein. The results are given as percentages of the ATP content present in the control before the stress (2.44 ± 0.06 pmol ATP/μg protein) and are presented as means of four experiments ± S.D.

mated 2 days after the stress and compared to the control. Results presented in Fig. 2 show an increased protection from 10^{-9} M to 10^{-7} M in naftidofuryl. Between 10^{-8} M and 10^{-5} M, the amounts of proteins were significantly higher ($P < 0.05$) than for the cells treated with TBHP alone. From 10^{-7} M to 10^{-5} M, a

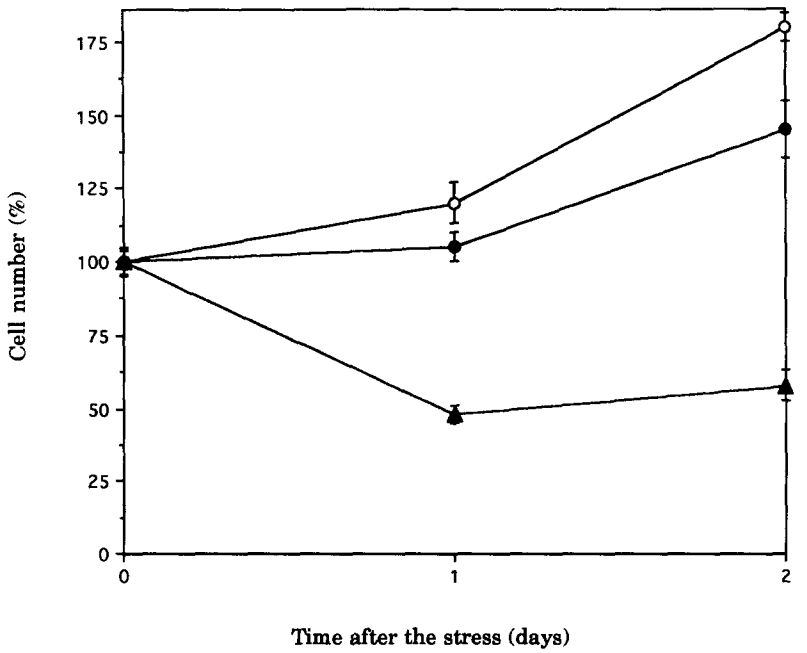

Fig. 6. Cell survival after a stress under TBHP. WI-38 fibroblasts at a density of 700 cells/cm^2 were stressed for 1 h under $5 \cdot 10^{-5}$ M TBHP diluted in PBS alone (▲) or PBS + 5 mM pyruvate (●). Cell numbers were determined at day 1 and 2 after the stress by countings and expressed in percentages of the cell numbers present before the incubation. Control: mean values of the mixed results obtained after an incubation for 1 h under PBS or PBS + 5 mM pyruvate (○). The results are given as mean values of four countings ±S.D. versus the time after the stress (days).

plateau effect is obtained. Naftidofuryl has been reported to stimulate the Krebs cycle [13,14] and to increase the glucose transport in cells [27].

3.5. Measurements of ATP concentrations

In order to assess the effect of these metabolites on the energetic potential of the cells, we directly measured the ATP content of these cells in various conditions. Cells were subcultivated as explained in Section 2 and, 24 h later, were stressed for 1 h under $5 \cdot 10^{-5}$ M TBHP diluted in PBS in the presence of 5 mM pyruvate + 1 mM malate, 5 mM D-glucose, 5 mM glutamate + 1 mM malate or 10^{-5} M naftidrofuryl, and the ATP content determined for each conditions.

First, a decrease between 40% and 50% in the ATP content of the cells was observed after a stress under TBHP alone, while it remained close to the control (from 90% to 100% of the control) in the presence of D-glucose or pyruvate-malate. Only a 25% decrease was observed in the presence of glutamate-malate. A small protection of 10% was observed in the presence of naftidrofuryl (Fig. 3.). These amounts of proteins were all significantly higher ($P < 0.05$) than for the cells treated with TBHP alone. When different concentrations of TBHP were used for the stress, the ATP content dropped but the protection obtained in the presence of pyruvate was significant ($P < 0.05$) in each case (Fig. 4). The evolution of the ATP content with increasing time under TBHP is shown in Fig. 5. A progressive decrease in the ATP content is observed while the presence of glucose keeps the level close to the initial value. The values obtained with glucose were significantly higher than those obtained without glucose ($P < 0.05$) except for a small but non-significant decrease after 15 min. The ATP quantities present in the medium just after the stress and in the washing medium were measured. Only 3% and 4% of the total ATP amount were respectively found in these media, excluding a high leakage during the stress.

The determination of the protein level 2 days after the stress cannot differentiate between a protection exerted on cell survival or on the mitosis. To discriminate between these different effects, we followed the evolution of the cell number after the stress. At day 1 after a stress under TBHP alone, a 30% cell loss was observed (Fig. 6) and a small recovery was obtained after the second day. If pyruvate was added to the medium, no cell loss was observed at day 1 after the stress ($P < 0.05$), and a 50% increase was observed between day 1 and day 2 after the stress. Pyruvate was added alone without malate, given it was shown above that pyruvate alone afforded a similar protection. The absolute number was, however, slightly lower than the cell number observed for the control incubated in PBS alone or in the presence of pyruvate without TBHP. In all these studies, the net observation was the protection afforded by the energetic metabolites on cell toxicity. In order to understand the effect of TBHP on cell division, an evaluation of the DNA synthesis was performed after the stress. [^{3}H]Thymidine was supplied to the cells between day 1 and day 2 after the stress and the proportion of labelled nuclei was expressed versus TBHP concentration (Fig. 7). A decrease in labelled nuclei was found with increasing TBHP concentrations. However, no significant difference was observed be-

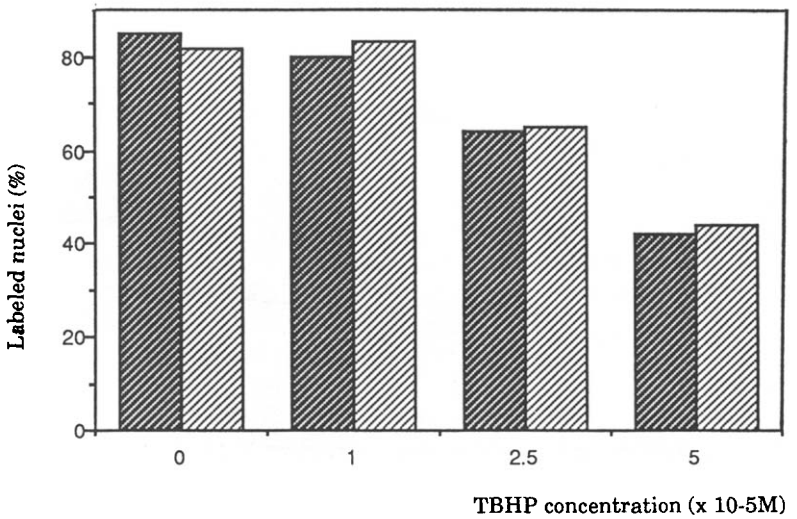

Fig. 7. Evolution of the mitotic index after stresses under TBHP. WI-38 fibroblasts at a density of 700 cells/cm^2 were stressed for 1 h under $5 \cdot 10^{-5}$ M TBHP diluted in PBS alone (dark columns) or PBS + 5 mM pyruvate (clear columns). Between day 1 and day 2 after the stress, cells were incubated with BME + S + [^{3}H]thymidine. After such an incubation, cells were fixed and autoradiography was performed as classically described by Cristofalo and Sharf [22]. Results are given as the percentages of labelled nuclei/total nuclei and are the mean values of two countings of 500 cells/flask.

tween cells stressed in the presence of pyruvate or not. Thus the arrest of the cell cycle does not seem to be affected by the presence of pyruvate. Similar observations have been made by Poot et al. [28] using cumene hydroperoxide. They found an accumulation of cells in G2 phase which was proportional to the stressing molecule concentration. The protection observed by pyruvate 1 day after the stress can then only be explained by an effect on cell toxicity which is well observed 1 day after the stress (Fig. 5), rather than a protection on the mitosis. After 1 day, in both cases, cell division is reinitiated, explaining the thymidine incorporation and the increase in cell number after 2 days.

4. Discussion

From the total free energy contained in metabolites entering the cellular catabolism, about 40% will be converted into the energy-rich phosphodiester bound to the ATP and the reducing NADPH. Free energy liberated in the catabolic reactions is required by cells to perform their various energy-requiring functions. One of the major properties of cells regarded as open systems is that they function at a minimum of entropy production, meaning that they optimize their overall energy required processes, taking into account their differentiation state and their interactions with the environment. Since the amount of available free energy is limited, a global regulation is taking place within the cell acting as a very regulated system, integrating the information and reacting accordingly [1].

Stresses induce an energy-requiring response from the cells and the level of energy is an essential parameter which will influence the cell resistance to the stress. The cell response for an increase in energy requirements in stressful conditions is demonstated by the fact that various stresses can either induce the expression of a glucose transporter [29], or trigger a reversible and specific translocation of the transporter protein from an (inactive) intracellular site to the plasma membrane [30]. Such a thermodynamic approach also predicts that the level of the cell metabolism will affect the cell survival after a stress. The results obtained in this work are in accordance with these predictions.

First, cells were submitted to stresses in conditions where their mitochondria were partly uncoupled, thus in conditions presumably lowering the ATP turnover compared to the numbers of electrons given by NADH and $FADH_2$. Stresses under TBHP or under ethanol showed a synergistic effect of cell mortality. So a decrease in the capacity to regenerate the ATP effectively leads to an increase in the cellular susceptibility to these stresses. Both molecules tested can kill the cells at the high concentrations [2,31], but by different mechanisms: TBHP is a source of free radicals reacting with many biological molecules such as proteins, fatty acids, and DNA [32], while ethanol disturbs fibroblast metabolism [33] but can also destabilize cell membranes or alter the calcium metabolism [34]. Given the fact that TBHP and ethanol have different targets, these results suggest that the synergistic effect of the uncoupling exist whatever the nature of the stress.

Secondly, the composition of the medium in which the stress is performed was changed. Vitamin E and albumin were found to have a positive effect on the cell protection. Of course, other elements present in the serum which were not tested could also protect against the free radicals. However, molecules related to the energetic metabolism were also found to be protective for the stress. These are D-glucose, pyruvate, and glutamate plus malate. Naftidrofuryl oxalate, a pharmacological stimulator of the energetic metabolism which has been reported to stimulate the Krebs cycle [13,14] and deoxyglucose uptake in cells [27], was also found to be protective.

To confirm that the metabolites tested effectively protected the cells by increasing the energy level of the cell, the amount of ATP in the presence of these molecules during a stress was measured. The results obtained after such measurements effectively showed that a parallel evolution exists between the ATP contents obtained just after the stress and the amount of proteins found 2 days after the stress. TBHP can have a direct effect on the production of ATP by mitochondria, since it can affect the potential of the inner mitochondrial membrane [35]. At very high TBHP concentrations, it is probable that such a decrease of potential is irreversible, which could explain that a protection by energetic metabolites is only found at low TBHP concentrations.

Comparing the ATP content in different cell types may not be significant: given their differentiation state, the requirement in energy of their functions, or their ATP turnover rate, various cell types may contain different ATP levels and nevertheless be healthy. However, if the ATP level is reduced within a given cell type, this may have highly significant consequences on cell survival in stressful conditions. Tribble et al. [36] tested the cytotoxicity of TBHP on cultured hepatocytes, concomitantly with a decrease in the oxygen pressure. The cytotoxicity of TBHP was found to increase with hypoxia. They also showed that lipid peroxidation was lower in such hypoxic conditions so that cell death was independent on lipid peroxidation. They concluded that the increase in TBHP cytotoxicity under hypoxia results from a decrease in the defence capacity of the cells due to a decrease in the capacity of energy production. Experiments performed with endothelial cells in the context of the ischemia-reperfusion toxicity of free radicals show the impact of the

cellular energetic level on the cell resistance to free radicals. When incubated under low O_2 pressure, endothelial cells display a lowered ATP level and become more susceptible to the free radicals produced by the activated xanthine oxidase-xanthine system. However, if a supplement of energy is given during hypoxia, the toxic effects of the free radicals is reduced. In such an experimental system, protection could be obtained either by lowering the level of the stress by using an inhibitor of xanthine oxidase or raising the antioxidant level, or also by giving the cell a higher turnover of its metabolism [37]. Moreover, it is known that hypoxic cells react by inducing the synthesis of proteins referred to as glucose and oxygen regulated proteins (GRPs and ORPs, respectively) [38].

Another cause of a lowering in free energy would be the presence of mutated DNA in some mitochondria, either in pathological conditions or in aging [39–41]. In stressful conditions where an excess of free energy production is necessary for cell survival, these deletions could have a more important effect than in normal conditions. This could partly explain the lower resistance of old cells to stress.

In conclusion, the results obtained in this work are in favour of the theoretical model of cells considered from a thermodynamic points of view, since it clearly shows that the level of the energetic metabolism modulates the intensity of the cell response to stress. This level of energy metabolism influences the survival of the cells. Since old cells have more and more difficulty in producing, and use a sufficient amount of, free energy in stressful conditions, these cells will not be able to cope with their environment and they will more rapidly degenerate and die [7].

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