ATP depletion inhibits glucocorticoid-induced thymocyte apoptosis

Claudio STEFANELLI*§, Francesca BONAVIDA*, Ivana STANIC*, Giovanna FARRUGGIA*, Elisabetta FALCIERI†‡, Iole ROBUFFO‡, Carla PIGNATTI*, Claudio MUSCARI*, Carmen ROSSONI*, Carlo GUARNIERI* and Claudio M. CALDARERA*

*Dipartimento di Biochimica 'G. Moruzzi', Università di Bologna, via Irnerio 48, I-40126 Bologna, Italy, †Istituto di Anatomia e Fisiologia, Università di Urbino, Italy, and ‡Istituto di Citomorfologia Normale e Patologica, CNR, Bologna and Chieti, Italy

In quiescent thymocytes, mitochondrial de-energization was not correlated to apoptotic death. In fact, thymocytes treated with oligomycin, a highly specific inhibitor of ATP synthase, alone or with atractyloside to block ATP translocation from the cytoplasm, were alive, even if their mitochondria were depolarized, as revealed by flow cytometry after Rhodamine 123 staining. Furthermore, oligomycin was a powerful inhibitor of apoptosis induced in rat thymocytes by dexamethasone and, to a lesser extent, by the calcium ionophore A23187 and etoposide, but was without effect when apoptosis was induced by staurosporine, and increased cell death in mitogen-treated thymocytes. The inhibition of apoptosis was confirmed by morphological criteria, inhibition of inter-nucleosomal DNA fragmentation and inhibition of the loss of membrane integrity. The anti-apoptotic effect of oligomycin in cells treated with A23187 or etoposide was correlated to the inhibition of protein synthesis, while inhibition of apoptosis induced by dexamethasone, already evident at an oligomycin concentration of 10 ng/ml, was instead strictly cor-

related to the effect exerted on the cellular ATP level. Thymocyte apoptosis triggered by dexamethasone was blocked or delayed by inhibitors of respiratory-chain uncouplers, inhibitors of ATP synthase and antioxidants: a lasting protection from dexamethasone-induced apoptosis was always correlated to a drastic and rapid reduction in ATP level (31–35 % of control), while a delay in the death process was characterized by a moderate decrease in ATP (73–82 % of control). Oligomycin inhibited the specific binding of radioactive corticosteroid to thymocyte nuclei, confirming the inhibitory effect of ATP depletion on glucocorticoid binding and suggesting that ATP depletion is a common mediator of the anti-apoptotic action of different effectors in glucocorticoid-induced apoptosis. In conclusion, the reported data indicate that ATP may act as a cellular modulator of some forms of apoptosis, depending on the death trigger, and that in quiescent cells the de-energization of mitochondria is not necessarily linked to apoptosis.

INTRODUCTION

Apoptosis is a multi-step process which may be induced by a wide variety of stimuli [1]. The death process may be divided into an induction phase and an effector phase, driving the cell to irreversible commitment to death, followed by the degradation phase, when characteristic apoptotic morphology is manifested [2].

Despite intensive research, the signal-transduction pathway(s) involved in cell suicide are still poorly understood. Several data support a role for oxidative mechanisms in the induction of apoptosis [3–5]. It has also been suggested that the *bcl-2* proto-oncogene, which has apoptosis-inhibiting capabilities, may function with an antioxidant mechanism [6]. In view of this, mitochondria should be considered of primary importance in programmed cell death (PCD); in fact the Bcl-2 protein is mainly located in the inner mitochondrial membrane [7], and mitochondria represent the principal site in which intracellular reactive oxygen species (ROS) are formed [8]. Actually, it has been suggested that apoptosis may be regarded as a mitochondrial catastrophe [2] because: (1) the reduction in mitochondrial potential constitutes an early step of lymphocyte apoptosis [9]; (2) alterations in mitochondrial structure and function characterize dexamethasone-induced thymocyte apoptosis [10]; and (3) inhibition of mitochondrial electron transport could induce apoptosis in several cell lines [11]. Conversely, other experi-

mental evidence argues against a general involvement of ROS and mitochondria in PCD. In fact, apoptosis may be induced in cells lacking functional mitochondria [12,13].

We observed that hypoxia may delay apoptosis in the prototypic system of dexamethasone-treated thymocytes [14]. Subsequently, we found that the effect of anoxic hypoxia could be mimicked by chemical hypoxia, obtained with inhibitors of cellular respiration. Here we report that oligomycin and other inhibitors of mitochondrial function are powerful inhibitors of dexamethasone-induced apoptosis in quiescent thymocytes, in contrast to their effect on tumour cells [11,15,16]. We conclude that mitochondrial de-energization is not a necessary step of apoptosis and that ATP level affects the sensitivity of thymocytes to glucocorticoid-induced apoptosis.

MATERIALS AND METHODS

Reagents

Dexamethasone, oligomycin and other inhibitors of mitochondrial respiration, as well as other biochemicals, were purchased from Sigma Chemical Co. The preparations of oligomycin used (90 % total oligomycin) were composed of a mixture of A, B and C forms (59 %, 21 % and 10 % respectively). D,L-[1-¹⁴C]Leucine (54 Ci/mol) was obtained from Amersham and [1,2-

Abbreviations used: PCD, programmed cell death; ROS, reactive oxygen species; PI, propidium iodide; LS1, small-angle light scatter; LS2, large-angle light scatter; LDH, lactate dehydrogenase; Tempo, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl; PBN, α -phenyl-*t*-butyl nitron; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TNF, tumour necrosis factor.

§ To whom correspondence should be addressed.

^3H]corticosterone (47 Ci/mmol) was from New England Nuclear. Propidium iodide (PI) and other dyes used in flow cytometry were products of Molecular Probes.

Cell culture

Thymocytes were isolated from 4–8-week-old male Wistar rats and diluted to a density of 20×10^6 cells/ml (unless otherwise stated) in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 10 mM Hepes, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. The cells were incubated in 5% $\text{CO}_2/95\%$ air. Cell viability was routinely evaluated by Trypan Blue exclusion. All the reagents to be tested were dissolved in PBS or ethanol, if water-insoluble, as 400-fold concentrated solutions (except *N*-acetylcysteine, which was directly dissolved in the medium), and 2.5 $\mu\text{l}/\text{ml}$ was added to the thymocyte suspensions. No effect of 0.25% PBS or ethanol on any of the parameters evaluated in the present study was observed.

DNA fragmentation

A quantitative assay of DNA fragmentation was carried out essentially as described by McConkey et al. [17]. At the end of incubation, the cells (2 ml/well) were collected, washed and then lysed in 10 mM Tris/HCl, pH 8/10 mM EDTA/0.2% (v/v) Triton X-100. Low-molecular-mass DNA was then separated from unfragmented DNA by centrifugation for 20 min at 13000 *g*, and the DNA content in each fraction was measured with diphenylamine reagent. The percentage of DNA fragmentation was defined as the ratio of the DNA content of the detergent-soluble supernatant obtained at 13000 *g* to the total DNA in the lysate.

To visualize DNA fragmentation, thymocytes (10×10^6 cells in 1 ml of medium) were collected after incubation, washed with ice-cold PBS, lysed in 10 mM Tris/HCl, pH 8/2 mM EDTA/1% (w/v) SDS and incubated overnight with 0.1 mg/ml proteinase K. The DNA was extracted with phenol/chloroform (1:1) and ethanol-precipitated in the presence of 0.5 M NaCl at -20°C . After quantification, 2 μg of each DNA sample was subjected to electrophoresis on a 1.2% agarose gel impregnated with ethidium bromide, and visualized under UV light.

Electron microscopy

At the end of the incubation, cells were washed in PBS, sedimented and quickly fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 h. After 1 h of post-fixation treatment with 1% OsO_4 in veronal buffer and ethanol dehydration, the pellets were embedded in araldite. Semi-thin sections, prepared to provide an overall light microscopy picture and to quantify apoptosis, were stained with 1% Toluidine Blue at 40°C . Thin sections, collected on 200 mesh nickel grids, were stained with uranyl acetate and lead citrate. The observations were carried out with a Zeiss 109 electron microscope at 80 kV.

Assessment of apoptosis, cell viability and mitochondrial potential by flow cytometry

Cell samples (10^6 cells/ml) were analysed using a Bryte HS flow cytometer equipped with a Xe–Hg lamp. The emitted fluorescence and small-angle and large-angle light scatter (LS1 and LS2 respectively) were analysed at a cell throughput rate of 500 cells/s. LS1 and LS2 were analysed using a linear scale, while emitted fluorescence was measured as reported below. Thymocytes were recognized by their scatter cytogram and the fluorescence of debris was excluded. PI was used in all the assays to

detect dead cells: cells which incorporated PI were considered dead, and an electronic gating on PI-positive cells was imposed in order to monitor apoptosis or mitochondrial activity of viable cells only.

Apoptosis was detected according to the method of Dive et al. [18] with minor modifications. Briefly, Hoechst 33258 and PI were dissolved in distilled water and added to cell suspensions to final concentrations of 10 μM and 32 μM respectively. Hoechst 33258 was added immediately before PI. After 1 min of staining, at least 5000 cells were analysed using a filter set to achieve an excitation light between 360 and 370 nm and to detect emission at between 390 and 490 nm for Hoechst 33258 and between 520 and 720 nm for PI. Results are represented as a two-dimensional frequency contour plot of LS1 versus log Hoechst 33258 blue fluorescence.

The mitochondrial potential was assessed with the dye Rhodamine 123 [19], counterstained by PI to detect dead cells in the population being examined. Rhodamine 123 (2.5 mM in ethanol) was added to the cells to achieve a final concentration of 5 μM , then the cells were kept for 15 min at 37°C . At 1 min before analysis 5 $\mu\text{g}/\text{ml}$ of PI was added to stain dead cells.

Flow cytometric analysis was performed using a filter set producing an excitation light between 470 and 490 nm and emission between 515 and 565 nm for Rhodamine 123 and between 590 and 720 nm for PI. The fluorescence was analysed using a logarithmic scale for Rhodamine 123 and a linear scale for PI. Fluorescence intensity, collected by a multichannel analyser, is reported as the mean channel of the fluorescence distribution.

Cell death

A quantitative assay of cell death was performed by flow cytometry, measuring the percentage of PI-incorporating cells, as described above, and by measuring lactate dehydrogenase (LDH) leakage into the medium from damaged cells [20]. To measure LDH release, thymocytes (1 ml/well) were incubated for 24 h, the cells were then collected by centrifugation and the fraction of LDH activity released from dead cells was measured spectrophotometrically in the supernatant. To obtain total LDH activity in the sample, another aliquot of cells treated in the same manner was frozen for 15 min at -80°C , quickly thawed at 37°C and vortexed in order to disrupt membrane integrity. The percentage of LDH release represents the fraction of LDH activity found in the medium, with respect to the overall enzyme activity.

Steroid binding to thymocyte nuclei

Specific and non-specific nuclear binding of radioactive corticosterone was determined by the procedure of Sloman and Bell [21]. Briefly, 100 μl samples of cell suspensions (5×10^7 cells) were incubated in triplicate for 60 min with 20 nM [$1,2\text{-}^3\text{H}$]corticosterone in the presence or absence of 10 μM non-radioactive dexamethasone. The cells were then lysed with 1.5 mM MgCl_2 . Nuclei were collected on Whatman GF/C glass-fibre filters and washed with 1.5 mM MgCl_2 , then the radioactivity in the filters was measured by liquid scintillation counting. Specific steroid binding by nuclei was determined as the difference in the level of radioactive steroid retained on the filters in the presence and absence of non-radioactive dexamethasone. The absolute values of nuclei-bound radioactivity varied from 10 to 45% among replicate experiments in which different thymocyte preparations were used. This difference was also noted in preliminary experiments in which the binding at equilibrium of

corticosterone to intact thymus cells was determined [22], and was ascribed to the fact that we used rats that were not adrenalectomized. However, the effect of oligomycin was very similar in all four experiments performed.

Other assays

For measuring ATP level, thymocytes (1 ml/well) were collected by centrifugation in an Eppendorf tube, 0.5 ml of chilled 0.6 M perchloric acid was added to the cells, and the samples were immediately frozen in liquid nitrogen. After thawing, the samples were centrifuged for 1 min at 17000 *g* and the supernatant was neutralized by adding a fixed amount of 6 M KOH/2 M KH₂PO₄ buffer (pH 6.25). ATP in the extracts was quantified by isocratic ion-pairing reverse-phase HPLC and UV detection [23]. To evaluate protein synthesis, the cells (1 ml/well) were incubated with 0.25 μ Ci of L-[1-¹⁴C]leucine. After 4 h, thymocytes were collected, washed twice with PBS and the radioactivity incorporated in the trichloroacetic acid-insoluble fraction was measured by liquid scintillation counting.

Data presentation

Each experimental point was derived from two separate wells each assayed in duplicate. All the data presented were obtained from experiments performed at least twice with comparable results. The data reported in the Figures and Tables are means $\pm$ S.D. obtained in the indicated number of separate determinations (if not otherwise stated). Data were compared by Student's *t*-test and were considered significantly different at *P* < 0.05.

RESULTS

Effects of oligomycin and dexamethasone on mitochondrial potential and cell death of rat thymocytes

In order to verify the hypothesis that ATP depletion could affect thymocyte apoptosis, we studied the effect of oligomycin, a highly specific inhibitor of ATP synthase. Our hypothesis was not consistent with the model hypothesizing that the fall in ATP and the consequent depolarization of the mitochondrial membrane are essential steps of apoptosis [2,5,9,10]; hence we first examined the effects of oligomycin and dexamethasone on mitochondrial potential and cell death. Rhodamine 123 was used to detect the mitochondrial potential of viable thymocytes after different treatments. Figure 1 reports the flow cytometric patterns obtained in a typical experiment. Rhodamine 123 was counterstained by PI (left column) to detect cells with permeabilized plasma membranes which were considered dead cells (region 2). The mitochondrial potential of viable cells (right column) was detected via an electronic gating on region 1, in order to exclude dead cells. After a 4 h incubation, both control and dexamethasone-treated cells showed a homogeneous distribution of mitochondrial potential, with a single detectable population. On the other hand, oligomycin, alone or together with dexamethasone, caused the appearance of a second cellular population, showing a fluorescence distribution characterized by a lower mean channel, indicating, as expected, the depolarization of the mitochondrial membrane. However, mitochondrial de-energization (right column) was not coupled with cell death (left column). This can be better appreciated by studying Table 1, which reports the quantitative data on cell death. Flow cytometric detection of PI-positive cells and apoptotic cells was performed after incubation for 7 h, as the permeabilization of the plasma membrane is not an early event in thymocyte apoptosis [10]. The percentage of dead cells was calculated on the basis of the

Rhodamine-123 fluorescence intensity

Figure 1 Flow cytometric detection of viability and mitochondrial potential of rat thymocytes exposed to different treatments

Thymocyte suspensions were exposed for 4 h to different treatments: (A, B) control cells; (C, D) 1 μ M dexamethasone; (E, F) 10 μ g/ml oligomycin; (G, H) dexamethasone plus oligomycin. At the end of the incubation, the cells were loaded with Rhodamine 123 and exposed to PI. Results are from a typical experiment where at least 5000 cells were analysed. On the left (A, C, E, G) are reported the two-parameter frequency contour plots of green fluorescence (Rhodamine 123 fluorescence intensity on the abscissa, logarithmic scale from 1 to 64) versus the red fluorescence of PI on the ordinate (channel number FL 2, in arbitrary units on a linear scale from 0 to 64). Two identified subpopulations are gated as region 1 (viable cells, PI negative) and region 2 (dead cells, PI positive). On the right (B, D, F, H) the univariate frequency histogram of Rhodamine 123 green fluorescence (channel number, logarithmic scale from 1 to 256) for thymocytes of region 1 is reported, i.e. the Rhodamine 123 fluorescence distribution of viable cells only, excluding PI. The scale of cell count (on the ordinate) is linear from 0 to 200.

number of cells falling in region 2, while the combination of Hoechst 33258 and PI was used to detect apoptotic cells [18]. Again PI-positive cells were considered dead, while apoptosis

Table 1 Oligomycin blocks thymocyte death that follows dexamethasone treatment

Thymocyte suspensions (20×10^6 cells/ml) were treated with $10 \mu\text{g/ml}$ oligomycin and/or with $1 \mu\text{M}$ dexamethasone, as indicated. Cell death was then measured by different methods. LDH leakage in the medium was determined after 24 h of incubation, while the percentages of cells including PI (PI positive) and of apoptotic cells were measured by flow cytometry after 7 h. Apoptosis was measured only in viable cells, excluding PI, and the indicated percentages of apoptotic cells represent the fraction of cells committed to apoptotic death, but still presenting membrane integrity. Results are means $\pm$ S.D. obtained for the number of determinations given in parentheses. * $P < 0.05$ compared with the corresponding control.

Treatment	LDH release (%)	PI-positive cells (%)	Apoptotic cells (%)
None (control)	21 ± 4 (32)	8 ± 3 (6)	13 ± 4 (6)
Oligomycin	27 ± 5 (6)	5 ± 2 (6)	9 ± 2 (6)
Dexamethasone	57 ± 5 (18)*	19 ± 3 (6)*	37 ± 6 (6)*
Dexamethasone + oligomycin	26 ± 4 (8)	7 ± 2 (6)	11 ± 3 (6)

Table 2 Effect of inhibitors of ATP synthesis and translocation on thymocyte death

Thymocyte suspensions were incubated for 24 h with the following treatments: $10 \mu\text{g/ml}$ oligomycin, 1 mM NaCN, $20 \mu\text{M}$ atractyloside, 20 mM 2-deoxyglucose. At the end of the incubation the cells were collected and LDH leakage from dead cells and DNA fragmentation were measured. Results are means $\pm$ S.D. obtained for the number of determinations given in parentheses. * $P < 0.05$ compared with the corresponding control.

Treatment	LDH release (%)	DNA fragmentation (%)
None (control)	21 ± 4 (32)	24 ± 4 (14)
Oligomycin	27 ± 5 (6)	23 ± 6 (6)
Oligomycin + atractyloside	22 ± 6 (6)	17 ± 5 (6)
Oligomycin + 2-deoxyglucose	67 ± 9 (6)*	10 ± 5 (6)*
NaCN	25 ± 4 (4)	20 ± 4 (4)
NaCN + atractyloside	20 ± 5 (4)	23 ± 6 (4)
NaCN + 2-deoxyglucose	70 ± 11 (4)*	9 ± 4 (4)*

was detected only in PI-negative cells. After 7 h, 38% of glucocorticoid-treated thymocytes were apoptotic (14% in controls). However, if oligomycin was administered together with dexamethasone, cell death was inhibited and only 11% of cells were apoptotic. Cell death was also evaluated after 24 h of incubation by measuring the amount of LDH leakage. As expected, dexamethasone largely increased LDH release to about 60% of total enzyme activity. Again, oligomycin inhibited the release of LDH in glucocorticoid-treated cells.

The mitochondrial transmembrane potential may be partially maintained by the import of cytoplasmic ATP into the mitochondrion [24]. The adenine nucleotide translocator is inhibited by atractyloside, and micromolar concentrations of this drug inhibit the importation of ATP into the mitochondria of cultured cells [25]. Thymocytes were then treated with $10 \mu\text{g/ml}$ oligomycin or 1 mM NaCN together with $20 \mu\text{M}$ atractyloside to inhibit ATP translocation, or 20 mM 2-deoxyglucose in order to block glycolysis (Table 2). Inhibition of mitochondrial respiration did not affect cell viability, but when the cells were incubated with oligomycin or cyanide together with 2-deoxyglucose, cell death was largely increased without DNA fragmentation, whereas the ATP level fell to less than 10% of control (results not shown). This indicates, as expected, that following inhibition of the respiratory chain, thymocytes depend on anaerobic ATP formation to survive and that inhibition of this pathway causes massive necrotic death. On the other hand, atractyloside (we

Figure 2 Oligomycin inhibits DNA fragmentation in dexamethasone-treated thymocytes

Thymocytes (20×10^6 cells/ml) were treated with $1 \mu\text{M}$ dexamethasone (dex) or the corresponding volume of ethanol in the presence or absence of $10 \mu\text{g/ml}$ oligomycin, and DNA fragmentation was detected. (A) Electrophoretic fractionation of DNA ($2 \mu\text{g}$) extracted from thymocytes, freshly prepared (lane 1) or incubated for 5 h without any treatment (control, lane 2), with dexamethasone (lane 3), and with dexamethasone plus oligomycin (lane 4). Lane 5 represents DNA size markers (*Bgl*I and *Hin*II pBR 328 digest). (B) DNA fragmentation was measured at the indicated times. Results are means $\pm$ S.D. of values obtained in two experiments (four determinations).

tested concentrations of $1\text{--}50 \mu\text{M}$ with similar results) did not enhance cell death, even when it was given together with oligomycin or cyanide (Table 2). Furthermore, atractyloside did not influence at all the inhibitory effect of oligomycin on dexamethasone-induced apoptosis (results not shown).

Oligomycin is a powerful inhibitor of thymocyte apoptosis induced by dexamethasone

The effect of oligomycin on thymocyte apoptosis was examined by other criteria. Agarose gel electrophoresis of DNA extracted from cells incubated for 5 h revealed that $1 \mu\text{M}$ oligomycin prevented the internucleosomal DNA fragmentation induced by $1 \mu\text{M}$ dexamethasone (Figure 2A). Inhibition of DNA fragmentation was long-lasting, and even after 24 h of incubation the percentage of fragmented DNA was very similar in control cells ($27 \pm 3\%$) and in dexamethasone-treated cells incubated with oligomycin ($30 \pm 4\%$). At this time, DNA fragmentation in cells treated with dexamethasone alone was about 90% (Figure 2B).

Apoptotic death is not always coupled with DNA laddering [26], therefore the effect of oligomycin on dexamethasone-induced

Figure 3 Effects of oligomycin on the morphology of dexamethasone-treated thymocytes

Electron microscopy of thymocytes incubated for 24 h under control conditions (A), after dexamethasone (B) and dexamethasone–oligomycin (C) treatment. Apoptotic nuclei in progressive stages of chromatin condensation (arrow) appear after dexamethasone treatment. Scale bar = 1 μ m. Dexamethasone and oligomycin were added to thymocyte suspensions at dosages of 1 μ M and 10 μ g/ml respectively.

Table 3 Effects of oligomycin on thymocyte apoptosis

Thymocytes (20×10^6 cells/ml) were incubated in the presence or absence of 10 μ g/ml oligomycin with 1 μ M dexamethasone, 0.5 μ M A23187, 10 μ M etoposide, 1 μ M staurosporine and 10 μ g/ml concanavalin A. DNA fragmentation and LDH release were measured after 24 h. Results are means $\pm$ S.D. for the number of determinations given in parentheses. * $P < 0.05$ compared with the corresponding treatment in the absence of oligomycin.

Treatment	DNA fragmentation (%)		LDH release (%)	
	– Oligomycin	+ Oligomycin	– Oligomycin	+ Oligomycin
Control	24 $\pm$ 4 (14)	23 $\pm$ 6 (6)	21 $\pm$ 4 (32)	27 $\pm$ 5 (6)
Dexamethasone	82 $\pm$ 8 (12)	28 $\pm$ 5 (6)*	57 $\pm$ 5 (18)	26 $\pm$ 4 (8)*
A23187	59 $\pm$ 7 (6)	36 $\pm$ 5 (4)*	56 $\pm$ 7 (4)	38 $\pm$ 5 (4)*
Etoposide	78 $\pm$ 9 (10)	39 $\pm$ 8 (4)*	61 $\pm$ 6 (4)	35 $\pm$ 5 (4)*
Staurosporine	63 $\pm$ 6 (10)	66 $\pm$ 7 (4)	51 $\pm$ 4 (4)	58 $\pm$ 8 (4)
Concanavalin A	43 $\pm$ 5 (4)	55 $\pm$ 8 (4)	38 $\pm$ 4 (4)	57 $\pm$ 6 (4)*

apoptosis was examined by electron microscopy (Figure 3). Control cells showed the characteristic ultrastructural aspect of rat thymocytes, with a high nuclear/cytoplasmic ratio and strongly condensed chromatin, while dexamethasone-treated cells appeared mostly apoptotic with nuclei entirely condensed. The apoptotic process was also correlated to a notably worse preservation of membrane and cytoplasmic components. Apoptotic morphology was completely inhibited when thymocytes were cultured in the presence of 10 μ g/ml oligomycin, which is the same dose reported to induce apoptosis in several cell lines [11], and dexamethasone–oligomycin-treated cells appeared closely comparable to the controls, showing only occasional apoptotic features. A weak swelling of membrane compartments was present, possibly as a consequence of the treatment. Nevertheless, no apoptosis could be identified.

Effect of oligomycin on cell death triggered by different stimuli

Thymocyte apoptosis was evoked by 0.5 μ M calcium ionophore A23187, 10 μ M etoposide or 1 μ M staurosporine. DNA fragmentation and cell lysis were measured in cells incubated for 24 h in the presence or absence of 10 μ g/ml oligomycin (Table 3).

Figure 4 Correlation between the anti-cytolytic action of oligomycin and the effect on ATP level and protein synthesis

(A) Thymocyte suspensions (20×10^6 cells/ml) were incubated for 24 h with 1 μ M dexamethasone (dex), 0.5 μ M A23187, 10 μ M etoposide or alone (control) in the presence of the indicated concentration of oligomycin. LDH released from dead cells was then measured. Each point represents the mean of two experiments. (B) Thymocytes were incubated in the presence of various concentrations of oligomycin. To measure the ATP level, cells were collected after 2 h of incubation and ATP was measured in acid extracts by HPLC. ATP content in control thymocytes was 0.60 ± 0.04 μ mol/ 10^6 cells ($n = 8$). The rate of protein synthesis was measured in cells incubated for 4 h with 0.25 μ Ci of L-[14 C]leucine. Control cells incorporated 186 ± 35 d.p.m./ 10^6 cells ($n = 10$). Values are the means of a number of determinations ranging from four to ten.

Oligomycin inhibited apoptosis also when the triggers were etoposide and A23187, even if the effect was less marked than with dexamethasone, but was without effect in staurosporine-

Table 4 Correlation between inhibition of dexamethasone-induced apoptosis and thymocyte ATP level

Thymocytes (20×10^6 cells/ml) were incubated without any treatment (Control) or with 1 μ M dexamethasone (Dex) in the absence or in the presence of the indicated compounds. DNA fragmentation was measured after 6 h and 24 h, LDH release was assayed after 24 h and ATP level was measured after 2 h. The absolute ATP content of control thymocytes was 0.60 ± 0.04 μ mol/ 10^6 cells ($n = 8$). The reported data are means $\pm$ S.D. obtained for the number of determinations given in parentheses. In the statistical analysis, the data for DNA fragmentation and LDH release were compared with the corresponding values obtained in cells treated with dexamethasone alone (Dex). The data for ATP content were compared with the control value (* $P < 0.05$).

Treatment	DNA fragmentation (%)		LDH release (%)	ATP (% of control)
	6 h	24 h	24 h	2 h
Control	10 $\pm$ 4 (10)*	24 $\pm$ 4 (14)*	22 $\pm$ 3 (32)*	100
Dex	36 $\pm$ 5 (10)	82 $\pm$ 8 (12)	57 $\pm$ 5 (18)	95 $\pm$ 6 (4)
Dex + 1 μ g/ml oligomycin	6 $\pm$ 4 (4)*	29 $\pm$ 7 (8)*	29 $\pm$ 4 (6)*	31 $\pm$ 5 (6)*
Dex + 5 μ g/ml rotenone	4 $\pm$ 3 (4)*	33 $\pm$ 5 (4)*	30 $\pm$ 5 (4)*	35 $\pm$ 8 (4)*
Dex + 5 μ g/ml antimycin A	7 $\pm$ 3 (4)*	33 $\pm$ 6 (4)*	27 $\pm$ 6 (4)*	34 $\pm$ 5 (4)*
Dex + 100 μ M 2,4-dinitrophenol	4 $\pm$ 2 (4)*	35 $\pm$ 7 (4)*	31 $\pm$ 4 (4)*	34 $\pm$ 8 (4)*
Dex + 1 mM NaCN	11 $\pm$ 6 (4)*	76 $\pm$ 11 (4)	53 $\pm$ 8 (6)	73 $\pm$ 8 (4)*
Dex + 10 mM Trolox	15 $\pm$ 5 (4)*	74 $\pm$ 10 (4)	55 $\pm$ 9 (4)	82 $\pm$ 5 (6)*
Dex + 100 μ M 1,10-phenanthroline	14 $\pm$ 6 (4)*	78 $\pm$ 16 (4)	50 $\pm$ 8 (4)	81 $\pm$ 6 (6)*
Dex + 5 mM Tempo	15 $\pm$ 6 (4)*	88 $\pm$ 10 (4)	62 $\pm$ 14 (4)	80 $\pm$ 6 (6)*
Dex + 5 mM PBN	32 $\pm$ 7 (4)	83 $\pm$ 11 (4)	53 $\pm$ 8 (4)	94 $\pm$ 5 (4)
Dex + 100 μ M tocopherol phosphate	37 $\pm$ 7 (4)	85 $\pm$ 9 (4)	62 $\pm$ 8 (4)	98 $\pm$ 7 (4)
Dex + 20 mM <i>N</i> -acetylcysteine	39 $\pm$ 7 (4)	76 $\pm$ 14 (4)	59 $\pm$ 7 (4)	95 $\pm$ 7 (4)

treated cells. Apoptosis induced by staurosporine was not decreased even when the cells were treated with both oligomycin and 50 μ M atractyloside (results not shown), confirming that mitochondrial function is not required for staurosporine-induced apoptosis [12]. However, oligomycin increased cell death in cells activated with the mitogen concanavalin A (10 μ g/ml).

Correlation between protection from apoptosis and ATP levels

For both calcium ionophore and etoposide, the anti-cytolytic effect of oligomycin was evident only at concentrations higher than 0.1 μ g/ml. On the other hand, a strong inhibition of dexamethasone-induced cytotoxicity was already evident when the oligomycin concentration was 10 ng/ml (Figure 4A).

The effect of oligomycin on the death of thymocytes treated with dexamethasone was strictly similar to the dose-effect on ATP level (Figure 4B). ATP was measured after a 2 h incubation, and its content began to decrease significantly at between 5 and 10 ng/ml oligomycin. In the presence of 10 ng/ml oligomycin, ATP level was 51 % with respect to control cells. The blocking of ATP formation is expected to inhibit energy-requiring processes, such as protein synthesis. Figure 4(B) also shows that the rate of protein synthesis was inhibited by oligomycin: a concentration of 100 ng/ml inhibited leucine incorporation by approx. 50 %.

During oxidative phosphorylation ATP synthesis is tightly coupled to electron transfer along the mitochondrial respiratory chain, and oligomycin, by inhibiting ATP formation, blocks the respiratory chain [27]. The effects of several compounds, which could mimic the action of oligomycin, were then compared for dexamethasone-induced apoptosis. We tested rotenone (5 μ g/ml), an inhibitor of complex I, antimycin A (10 μ g/ml), an inhibitor of complex III, NaCN (1 mM), which binds to cytochrome oxidase, and the uncoupler 2,4-dinitrophenol (100 μ M). All the cited treatments were expected to decrease ATP formation. Thymocytes were incubated with dexamethasone and the aforementioned agents, then we measured DNA fragmentation after 6 and 24 h of incubation, LDH release after 24 h and ATP levels after 2 h. As shown in Table 4, rotenone, antimycin A and 2,4-dinitrophenol blocked apoptosis similarly to oligomycin. However, cyanide inhibited DNA fragmentation at 6 h,

but not after 24 h; also, LDH release after 24 h of incubation was not inhibited by cyanide.

Since the respiratory chain is considered the major source of cellular ROS [8], the effect of some antioxidants on the same parameters was also tested. We used 20 mM *N*-acetylcysteine, 0.1 mM tocopherol phosphate, the spin traps 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (Tempo, 5 mM) and α -phenyl-*t*-butyl nitron (PBN, 5 mM), 0.1 mM 1,10-phenanthroline, a transition-metal chelator, and 10 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water-soluble analogue of vitamin E. *N*-Acetylcysteine, tocopherol phosphate and PBN did not protect thymocytes from dexamethasone-induced apoptosis at any time. 1,10-Phenanthroline, Trolox and Tempo inhibited DNA fragmentation after 6 h of incubation, according to previous reports [28–30], but none of these compounds inhibited DNA fragmentation or cell lysis in cells incubated for 24 h. A prolonged protection from dexamethasone-induced apoptosis was always correlated to a drastic reduction in ATP level (31–35 % of control), while a delay in the process was characterized by a moderate decrease in ATP (73–82 % of control).

Oligomycin inhibits the binding of corticosteroid hormone to thymocyte nuclei

Resistance to the cytolytic effect of dexamethasone progressively decreased when oligomycin was added to the culture medium more than 1 h after dexamethasone treatment. When ATP synthesis was inhibited later than 3 h after the addition of dexamethasone, no protective effect was observed (Figure 5A), suggesting that oligomycin inhibits an early event in dexamethasone-induced apoptosis.

It was discovered several years ago that ATP level could influence the binding of corticosteroids to thymus cells [22]. Therefore we tested the effect of oligomycin on the specific binding of [$1,3\text{-}^3\text{H}$]corticosterone to thymocyte nuclei. Oligomycin inhibited specific steroid binding in a concentration-dependent manner (Figure 5B). Non-specific binding, measured as radioactivity bound to nuclei in the presence of a large excess of non-radioactive dexamethasone, was not significantly affected by oligomycin.

Figure 5 ATP depletion blocks an early event in glucocorticoid-induced apoptosis; oligomycin inhibits specific corticosteroid binding to thymocyte nuclei

(A) Cell suspensions (20×10^6 cells/ml) were treated with $1 \mu\text{M}$ dexamethasone, then oligomycin was added after different times ranging from 0 h (oligomycin added together with the glucocorticoid) to 6 h. The overall incubation time was 24 h, then thymocytes were collected and the percentages of DNA fragmentation and LDH release were measured. The lower and upper horizontal lines represent the mean values measured in thymocytes that were incubated without any treatment (control) or in thymocytes treated with dexamethasone alone, without oligomycin addition (dex). Results are means $\pm$ S.D. of four determinations. (B) Aliquots of $100 \mu\text{l}$ of thymocyte suspensions (5×10^6 cells/ml) were incubated for 60 min with 20 nM radioactive corticosterone together with the indicated concentration of oligomycin, in the presence or absence of $10 \mu\text{M}$ dexamethasone. Nuclear binding was determined using a filter assay [28]. Specific binding of steroid by nuclei was determined as the difference in the level of radioactive steroid retained on the filters in the presence (non-specific binding) and absence (total binding) of dexamethasone. In control cells incubated without oligomycin the specific and non-specific radioactivity bound to nuclei (taken as 100%) were 71 ± 28 and 14 ± 7 d.p.m./ 10^6 cells respectively. Results are means $\pm$ S.D. of triplicate determinations obtained in a representative experiment.

DISCUSSION

Recently, growing attention has focused on the possible role of mitochondria in apoptosis. Several of the published studies deal with the cytotoxic action of tumour necrosis factor (TNF) and reach opposite conclusions. While some groups reported that the TNF-mediated production of mitochondrial ROS is responsible for apoptosis in target cells [31,32], other researchers presented evidence against the involvement of mitochondria in TNF action [13,33]. In a different experimental system, it was suggested that the reduction of mitochondrial transmembrane potential is an irreversible early step in the cascade of events accompanying PCD of lymphoid cells *in vivo* [9]. The same authors proposed a model where the drop in mitochondrial potential was accompanied by superoxide radical generation by an uncoupled respiratory chain, and the ROS formed could in turn participate in the effector phase of PCD [2,5]. As a consequence, the mere loss of mitochondrial function was implicated in the induction of

apoptosis [5], in line with previous findings that blockade of the respiratory chain with mitochondrial inhibitors induced apoptosis in several cell lines [11] and that the drop in mitochondrial membrane potential was an early event in other models of apoptotic death [34]. In the prototypic model of dexamethasone-induced thymocyte apoptosis, Petit et al. stated that mitochondrial membrane depolarization is a very early event, preceding DNA and plasma membrane alterations [10]. On the other hand, in the same experimental model, others reported that mitochondrial potential and structure are preserved during the early phases of apoptosis [35,36].

Our data indicate that mitochondrial de-energization does not trigger apoptotic death: thymocytes depleted of ATP by treatment with oligomycin died as control thymocytes, even if their mitochondria were largely impaired. To assess mitochondrial potential we used Rhodamine 123, a dye widely used for this purpose [19]. We did not detect alterations in cells incubated for 4 h with dexamethasone, which were abundantly apoptotic. On the other hand, a cell population with decreased mitochondrial potential was evident in thymocytes treated with oligomycin, which were alive. As the depolarization of mitochondria is not coupled to cell death, the impairment of mitochondrial function [5,9,10] and the decrease in ATP content that follow glucocorticoid treatment of thymocytes [37] have to be considered secondary effects of these hormones, and not mediators of apoptosis [2,5,9,10]. We also confirmed that some forms of apoptosis do not require mitochondrial function [12], since apoptosis induced by staurosporine was not affected by oligomycin alone or together with atractyloside.

Oligomycin inhibits apoptosis triggered by dexamethasone, calcium ionophore and etoposide. The anti-apoptotic effect in A23187- and etoposide-treated cells paralleled the inhibitory effect of oligomycin on protein synthesis, which is required for the action of these drugs as well as glucocorticoids [38,39]. On the other hand, the inhibition of apoptosis induced by dexamethasone, evident at very low concentrations of oligomycin, is correlated to the effect on ATP level (Figure 4). This correlation is an extension of an observation made many years ago. In pioneering work, Munck noted that some metabolic actions of glucocorticoids on thymic lymphocytes were abolished by anaerobiosis [40]. Subsequently it was shown that specific glucocorticoid binding to thymocytes depends on the availability of oxygen [22] and that the loss or acquisition of hormone-binding capacity paralleled changes in the level of cellular ATP [21,41,42]. Glucocorticoid binding was inhibited by treatments decreasing ATP, and in ATP-depleted cells the glucocorticoid receptor was bound to the nuclei in an inactive form which cannot bind its ligand [42]. These data were the first to suggest the influence of phosphorylation/dephosphorylation processes in the regulation of glucocorticoid receptor function [43]. We have confirmed the inhibition of glucocorticoid binding to ATP-depleted cells using oligomycin, a specific inhibitor of ATP synthase: oligomycin inhibits specific nuclear binding of glucocorticoid hormone and consequently blocks the principal effect of glucocorticoids on thymocytes, i.e. induction of apoptosis. Similarly, other ATP-decreasing treatments known to inhibit glucocorticoid binding to thymocytes, such as oxygen deprivation and 2,4-dinitrophenol [21,22,41,42], affect dexamethasone-induced thymocyte death ([14]; Table 4). Oligomycin and respiratory-chain inhibitors that greatly reduced the ATP level caused a long-lasting inhibition of glucocorticoid-induced apoptosis. Other treatments which decreased ATP by about 20% inhibited apoptosis, but their effect was transient.

Our data strongly suggest that mitochondrial inhibitors do not block dexamethasone-induced apoptosis by inhibiting mito-

chondrial ROS formation, but by decreasing the ATP level. This conclusion is supported by the fact that inhibitors of respiratory complexes, uncouplers and ATP synthase inhibitors have the same inhibitory effect, although they can increase the formation of radical species in mitochondria [44–46]. Antioxidants which delayed apoptosis also caused a decrease in ATP level (Table 4), suggesting that ATP depletion may be a common mediator of different effectors inhibiting the cytolytic action of glucocorticoids. The relatively small decrease in ATP level caused by antioxidants is expected to influence only partially the effect of glucocorticoids, and could explain their transient effect. Our findings do not exclude the involvement of oxidative mechanisms in apoptosis, since some antioxidants may inhibit apoptotic death triggered by stimuli other than dexamethasone [28,30,47] but do not support a role of oxidative stress in the induction of apoptosis, since only antioxidants decreasing ATP levels affected cell death. Antioxidants that inhibit apoptotic death might act via indirect mechanisms [48,49], interfering with some redox-regulated process within the cell or at the level of the plasma membrane. ATP depletion could also influence cell death by means of other mechanisms in addition to the inhibitory effect on glucocorticoid binding, such as inhibition of trafficking between nucleus and cytosol [50], interference at the transcriptional level [51] or inhibition of ATP-dependent proteases [52,53]. Interestingly the 20 S proteasome has recently been shown to play an important role in thymocyte apoptosis [54]. An AMP-activated kinase has been described which is activated when cellular ATP levels are depleted and when AMP rises [55,56], but to our knowledge it has not been implicated in apoptosis.

Rotenone, antimycin A and oligomycin were reported to induce an apoptotic response in cell lines at concentrations similar to those used in the present study [11]. The apoptotic action of mitochondrial inhibitors was ascribed to a direct effect on mitochondria [11], but following the decrease in ATP content, protein synthesis is inhibited, and it is well known that protein synthesis inhibitors are powerful inducers of apoptosis in several cell lines [57]. On the other hand, the inhibition of protein synthesis is known to inhibit [38] or, at least, delay [58] thymocyte apoptosis induced by stimuli requiring new proteins for their action. An anti-apoptotic action of some metabolic inhibitors has also been reported by others [36,59]. Alternatively it could be speculated that quiescent, non-proliferating thymocytes may survive with glycolytic ATP formation, and thymocytes can easily adapt to this condition [60]; however, rapidly dividing cells in culture continuously require energy, and ATP depletion could induce apoptosis. Accordingly, oligomycin increased cell death in mitogen-treated thymocytes. It would be interesting to compare the effect of ATP depletion in other forms of thymocyte activation, for example after anti-CD3 treatment.

In contrast with our results, in leukaemia/lymphoma cells, mitochondrial inhibitors restored the sensitivity to dexamethasone of resistant cell lines [15], which supports a difference in the response to ATP depletion between tumour and normal cells. However this discrepancy could also be explained by the finding that the activation of glucocorticoid receptor to the steroid-binding form is inhibited not only by low ATP, but even more by high ATP levels [61]. Cancer cells have ATP levels higher than quiescent untransformed cells, and inhibition of the respiratory chain would decrease ATP to the level of normal cells, permitting receptor activation.

It is intriguing that dexamethasone was found to protect cultured hepatocytes against cell killing by respiratory-chain inhibitors [62], according to the notion that glucocorticoids exert several opposite effects on liver and lymphoid tissues.

Results that correlate with ours have recently been reported in

a different experimental system [63]: in L929 cells the blockade of mitochondrial respiration decreased the TNF-binding capacity, which was correlated to the ATP level, and protected the cells from cytolysis. The similar cytoprotective effect of ATP depletion in TNF- and dexamethasone-induced cell death suggests that ATP could be involved in the regulation of some receptor-mediated cytotoxic mechanisms. In fact, a relatively small decrease in ATP (20 %) also affects thymocyte response to glucocorticoids, albeit transiently (Table 4), and it could be hypothesized that conditions leading to ATP depletion may influence cell sensitivity to external death stimuli, such as glucocorticoids or TNF, by modifying the receptor-binding capability.

A central role of intracellular ATP in the control of cell death has been proposed [64]. Our data strongly support a modulatory function of ATP in apoptosis, but they only partially fit with the proposed model which hypothesizes that cells can undergo apoptosis only if ATP is available, while ATP depletion shifts the death mechanism to necrosis [64]. The finding that ATP depletion protects thymocytes against apoptosis induced by dexamethasone, but not by staurosporine, and increases cell death in mitogen-activated cells, suggests that ATP does not play a unique role in the apoptotic process, but may act as a cellular modulator, depending on the cell cycle and the stimulus.

This research was supported by grants from CNR and the Ministry of University and Scientific Research (MURST). The authors are grateful to Dr. M. Columbaro for kind assistance in specimen preparation for electron microscopy, and to Mr. G. Negroni for excellent technical assistance.

REFERENCES

- 1 Arends, M. J. and Wyllie, A. H. (1991) *Int. Rev. Exp. Pathol.* **32**, 223–254
- 2 Kroemer, G., Petit, P., Zamzami, N., Vayssiere, J. L. and Mignotte, B. (1995) *FASEB J.* **9**, 1277–1287
- 3 Buttkie, T. M. and Sandstrom, P. A. (1994) *Immunol. Today* **15**, 7–10
- 4 Slater, A. F. G., Nobel, C. S. I. and Orrenius, S. (1995) *Biochim. Biophys. Acta* **1271**, 59–62
- 5 Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D., Macho, A., Hirsch, T., Susin, S. A., Petit, P. X., Mignotte, B. and Kroemer, G. (1995) *J. Exp. Med.* **182**, 367–377
- 6 Hockenbery, D. M., Oltvai, Z. N., Yin, X.-M., Millian, C. L. and Korsmeyer, S. J. (1993) *Cell* **75**, 241–251
- 7 Korsmeyer, S. J., Yin, X.-M., Oltvai, Z. N., Veis-Novack, D. J. and Linette, G. P. (1995) *Biochim. Biophys. Acta* **1271**, 63–66
- 8 Ernster, L. (1986) *Chem. Scripta* **25**, 525–534
- 9 Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J. L., Petit, P. X. and Kroemer, G. (1995) *J. Exp. Med.* **181**, 1661–1672
- 10 Petit, P. X., Lecoq, H., Zorn, E., Dauter, C., Mignotte, B. and Gougeon, M. L. (1995) *J. Cell. Biol.* **130**, 157–167
- 11 Wolvetang, E. J., Johnson, K. L., Krauer, K., Ralph, S. J. and Linnane, A. W. (1994) *FEBS Lett.* **339**, 40–44
- 12 Jacobson, M. D., Burne, J. F., King, M. P., Miyashita, T., Reed, J. C. and Raff, M. C. (1993) *Nature (London)* **361**, 365–369
- 13 Gamen, S., Anel, A., Montoya, J., Marzo, I., Pineiro, A. and Naval, J. (1995) *FEBS Lett.* **376**, 15–18
- 14 Stefanelli, C., Stanic', I., Bonavita, F., Muscare, C., Pignatti, C., Rossoni, C. and Caldarera, C. M. (1995) *Biochem. Biophys. Res. Commun.* **212**, 300–306
- 15 Smets, L. A., Van den Berg, J., Acton, D., Top, B., Van Rooij, H. and Verwijns-Janssen, M. (1994) *Blood* **84**, 1613–1619
- 16 Van den Borget, C., Dontje, B. H. T., Melis, T. E., Van der Veen, C. and Kroon, A. M. (1988) *Biochim. Biophys. Acta* **972**, 302–310
- 17 McConkey, D. J., Nicotera, P., Hartzell, P., Bellomo, G., Wyllie, A. H. and Orrenius, S. (1989) *Arch. Biochem. Biophys.* **269**, 365–370
- 18 Dive, C., Gregory, C. D., Phipps, D. J., Evans, D. L., Milner, A. E. and Wyllie, A. H. (1992) *Biochim. Biophys. Acta* **1133**, 275–285
- 19 Waggoner, A. S. (1990) in *Flow Cytometry and Sorting* (Melamed, M. R., Lindmo, T. and Mendelshon, M. L., eds.), pp. 209–225, Wiley-Liss, New York
- 20 Decker, T. and Lohmann-Matthes, M. L. (1988) *J. Immunol. Methods* **15**, 61–69
- 21 Sloman, J. C. and Bell, P. A. (1976) *Biochim. Biophys. Acta* **428**, 403–413
- 22 Munck, A. and Brinck-Johnsen, T. (1968) *J. Biol. Chem.* **243**, 5556–5565

- 23 Sellevold, O. F. M., Jynge, P. and Aarstad, K. (1986) *J. Mol. Cell. Cardiol.* **26**, 1513–1524
- 24 Skowronek, P., Haferkamp, P. O. and Rödel, G. (1992) *Biochem. Biophys. Res. Commun.* **187**, 991–998
- 25 Akerboom, T. P. M., Bookelman, H. and Tager, J. M. (1977) *FEBS Lett.* **74**, 50–54
- 26 Cohen, G. M., Sun, X. M., Snowden, R. T., Dinsdale, D. and Skilleter, N. (1992) *Biochem. J.* **286**, 331–334
- 27 Lehninger, A. L., Nelson, D. L. and Cox, M. M. (1993) *Principles of Biochemistry*, 2nd edn., Worth Publishers, New York
- 28 Wolfe, J. T., Ross, D. and Cohen, G. M. (1994) *FEBS Lett.* **352**, 58–62
- 29 Forrest, V. J., Kang, Y. H., McClain, D. E., Robinson, D. H. and Ramakrishnan, N. (1994) *Free Radical Biol. Med.* **16**, 675–684
- 30 Slater, A. F. G., Nobel, C. S., Maellaro, E., Bustamante, J., Kimland, M. and Orrenius, S. (1995) *Biochem. J.* **306**, 771–778
- 31 Hennet, T., Richter, C. and Peterhans, E. (1993) *Biochem. J.* **289**, 587–592
- 32 Goossens, V., Grooten, J., De Vos, K. and Fiers, W. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8115–8119
- 33 O'Donnell, V. B., Spycher, S. and Azzi, A. (1995) *Biochem. J.* **310**, 133–141
- 34 Vayssiere, J. L., Petit, P. X., Risler, Y. and Mignotte, B. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11752–11756
- 35 Cossarizza, A., Kalashnikova, G., Grassilli, E., Chiappelli, F., Salvioli, F., Capri, M., Barbieri, D., Troiano, L., Monti, D. and Franceschi, C. (1994) *Exp. Cell Res.* **214**, 323–330
- 36 Tiso, M., Gangemi, R., Bargellesi Severi, A., Pizzolitto, S., Fabbì, M. and Risso, A. (1995) *Am. J. Pathol.* **147**, 434–444
- 37 Nordeen, S. K. and Young, D. A. (1976) *J. Biol. Chem.* **251**, 7295–7303
- 38 Wyllie, A. H., Morris, R. G., Smith, A. L. and Dunlop, D. (1984) *J. Pathol.* **142**, 67–77
- 39 Walker, P. R., Smith, C., Youdale, T., Lablanc, J., Whitfield, J. F. and Sikorska, A. (1991) *Cancer Res.* **51**, 1078–1085
- 40 Munck, A. (1968) *J. Biol. Chem.* **243**, 1039–1042
- 41 Bell, P. A. and Munck, A. (1973) *Biochem. J.* **136**, 97–107
- 42 Mendel, D. B., Bodwell, J. E. and Munck, A. (1986) *Nature (London)* **324**, 478–480
- 43 Moudgil, V. K. (1990) *Biochim. Biophys. Acta* **1055**, 243–258
- 44 Boveris, A., Canedas, E. and Stoppani, A. O. M. (1976) *Biochem. J.* **156**, 435–444
- 45 Takeshige, K. and Minakami, S. (1979) *Biochem. J.* **180**, 129–135
- 46 Konstantinov, A. A., Peskin, A. V., Popova, E. Y., Khomutov, G. B. and Ruuge, E. (1987) *Biochim. Biophys. Acta* **894**, 1–10
- 47 McClain, D. E., Kalinich, J. F. and Ramakrishnam, N. (1995) *FASEB J.* **9**, 1345–1354
- 48 Yan, C. Y. I., Ferrari, G. and Greene, L. A. (1995) *J. Biol. Chem.* **270**, 26827–26832
- 49 Jacobson, M. D. (1996) *Trends Biochem. Sci.* **21**, 83–86
- 50 Finch, R. A. and Chan, P. K. (1996) *Biochem. Biophys. Res. Commun.* **222**, 553–558
- 51 Jiang, Y. and Gralla, J. D. (1995) *J. Biol. Chem.* **270**, 1834–1839
- 52 Rivett, A. J. (1989) *Biochem. J.* **263**, 625–633
- 53 Palombella, V. J., Rando, O. J., Goldberg, A. L. and Maniatis, T. (1994) *Cell* **78**, 773–785
- 54 Grimm, L. M., Goldberg, A. L., Poirier, G. G., Schwartz, L. M. and Osborne, B. A. (1996) *EMBO J.* **15**, 3835–3844
- 55 Corton, A., Gillespie, J. G. and Hardie, D. G. (1994) *Curr. Biol.* **4**, 315–324
- 56 Stapleton, D., Mitchelhill, K. I., Gao, G., Widmer, J., Michell, B. J., Teh, T., House, C. M., Fernandez, C. S., Cox, T., Witters, L. A. and Kemp, B. E. (1996) *J. Biol. Chem.* **271**, 611–614
- 57 Kochi, S. H. and Collier, R. J. (1993) *Exp. Cell Res.* **208**, 296–302
- 58 Chow, S. C., Peters, I. and Orrenius, S. (1995) *Exp. Cell Res.* **216**, 149–159
- 59 Kaufmann, S. H. (1989) *Cancer Res.* **49**, 5870–5878
- 60 Guppy, M., Greiner, E. and Brand, K. (1993) *Eur. J. Biochem.* **212**, 95–99
- 61 Sando, J. J., La Forest, A. C. and Pratt, W. B. (1979) *J. Biol. Chem.* **254**, 4772–4778
- 62 Pastorino, J. G., Wilhelm, T. J., Glascott, P. A., Kocsis, J. J. and Farber, J. L. (1995) *Arch. Biochem. Biophys.* **318**, 175–181
- 63 Sanchez-Alcazar, J. A., Hernandez, I., De la Torre, M. P., Garcia, I., Santiago, E., Munoz-Yague, M. T. and Solis-Herruzo, J. A. (1995) *J. Biol. Chem.* **270**, 23944–23950
- 64 Richter, C., Schweizer, M., Cossarizza, A. and Franceschi, C. (1996) *FEBS Lett.* **378**, 107–110