Interleukin-3 Withdrawal Induces an Early Increase in Mitochondrial Membrane Potential Unrelated to the Bcl-2 Family

ROLES OF INTRACELLULAR pH, ADP TRANSPORT, AND F₀F₁-ATPase*

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Cytokines such as interleukin-3 (IL-3) promote the survival of hematopoietic cells through mechanisms that are not well characterized. Withdrawal of IL-3 from an IL-3-dependent pro-B cell line induced early stress-related events that preceded cell death by more than 40 h. Intracellular pH rose above pH 7.8, peaking 2–3 h post-IL-3 withdrawal, and induced a transient increase in mitochondrial membrane potential (ΔΨₘ) detected using several different dyes. Similar events were observed following IL-7 withdrawal from a different dependent cell line. Bcl-2, Bax, and caspases were unrelated to these early events. Intracellular alkaline pH inhibited the mitochondrial import of ADP, which would limit ATP synthesis. Total cellular ATP sharply declined during this early period, presumably as a consequence of suppressed ADP import. This was followed by an increase in reduced pyridine nucleotides. The transient increase in ΔΨₘ was blocked by oligomycin, an inhibitor of F₀F₁-ATPase that may have undergone reversal caused by the abnormal ADP-ATP balance within mitochondria. These findings suggest a novel sequence of early events following trophic factor withdrawal in which alkaline pH inhibits ADP import into mitochondria, reversing the F₀F₁-ATPase, which in turn consumes ATP and pumps out protons, raising ΔΨₘ.

Interleukin (IL)1-3 is a cytokine that acts on early bone marrow-derived hematopoietic precursors, inducing their growth and differentiation into cells of the myeloid, lymphoid, and erythroid lineages (1). Withdrawal of IL-3 from dependent cell lines leads to cell death, with the activation of caspases and DNA fragmentation as late events (2). Inhibition of cell death is dependent on the membrane polarization and the concentrations of ADP and ATP in a thermodynamic balance (16). In addition, the metabolic changes that serve as initiating conditions for cytokine withdrawal-induced death are not fully understood.

Mitochondria play an essential role in many types of apoptotic death (2) by releasing cytochrome c, which activates the caspase cascade (6). In some studies, cytochrome c release preceded the loss of ΔΨₘ (7), whereas other studies reported the reverse, cytochrome c release following mitochondrial swelling, outer membrane rupture, and loss of ΔΨₘ (8, 9). Mitochondrial swelling is related to formation of the permeability transition (PT) pore, a complex that contains the voltage-dependent anion channel (VDAC), and the adenine nucleotide translocator (ANT) (9, 10) in addition to other cytosolic and mitochondrial proteins. The Bcl-2 family of anti- and pro-apoptotic proteins are modulators of these processes (11, 12). However, mitochondrial swelling, loss of ΔΨₘ and induction of the PT pore were all reversible events in osteosarcoma cells (13). Likewise in neuronal apoptosis, mitochondrial membrane depolarization was not fatal (14) nor was it a factor in the death of HL-60 cells induced by actinomycin-D, etoposide, or staurosporine (15). The progression to cell death, therefore, depends on the integration of mitochondrial processes that cannot be readily dissected.

Mitochondria produce energy in the form of ATP through oxidative phosphorylation, a process coupled to electron transport. Enzymes of the electron transport chain catalyze the transfer of electrons from NADH to O₂. The energy released through electron transport drives protons across the mitochondrial inner membrane, generating an electrochemical gradient. This distribution of protons produces both a pH gradient (with alkaline pH in the matrix and neutral pH in the cytosol) and a voltage gradient across the inner membrane or ΔΨₘ, the result of a net outflow of positive ions (with negative charges inside and positive charges outside; Ref. 16). The electrochemical proton gradient across the membrane of a respiring mitochondrion generates a potential of 200 mV, of which 60 mV results from the pH gradient (about one pH unit; Ref. 16).

The energy of this powerful electrochemical gradient drives the catalytic activity of the F₀F₁-ATPase, a member of the F class of ATP-powered proton pumps. Transport of protons back into the mitochondrial matrix through the F₀ component of the F₀F₁-ATPase drives the conversion of ADP to ATP, which is then exported to the cytosol in exchange for ADP by ANT. In reverse, the F₀F₁-ATPase can use the energy of ATP hydrolysis to pump protons out of the matrix through the inner membrane, increasing the ΔΨₘ.

F₀F₁-ATPase can therefore synthesize or consume ATP, depending on the membrane polarization and the concentrations of ADP and ATP in a thermodynamic balance (16). In addition,
a small inhibitory protein, IF1, which modulates the ATPase activity and proton conductance (17, 18), is thought to confer additional regulation. IF1 is pH-sensitive, increasing its inhibitory activity by binding the F_{1},F_{0}-ATPase in an acidic environment (19, 20).

In the current study, we examine aspects of mitochondrial bioenergetics within the first few hours following withdrawal of IL-3 from a dependent cell line. We identify a novel pathway in which intracellular alkalization induces mitochondrial hyperpolarization and total cellular ATP loss, which represent significant metabolic stresses to the cell and set the stage for later events in apoptotic cell death.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—**The IL-3-dependent murine pro-B cell line, FL5.12A (a kind gift from James A. McCubrey, East Carolina University, Greenville, SC), was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies). The IL-7-dependent thymocyte cell line D1 was previously described (21). The IL-3-dependent murine pro-B cell lines, FL5.12, overexpressing Bel-2, Bel-X, and the control neomycin vector were previously described (22, 23). The wild-type and Bax-mutant FL5.12 cell lines (maintained in medium containing 1 μM nigericin) were purchased from Life Technologies. Bcl-2, Bcl-XL, and the control neomycin vector were previously described (22, 23). The wild-type and Bax-deficient lines, FL5.12, overexpressing Bcl-2, Bcl-XL, and the control neomycin vector were purchased from Life Technologies.

**Chemicals—**MitoTracker Red CM-H2XROS (Molecular Probes), rotenone (Sigma), 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (Sigma), 1 μM nigericin (Molecular Probes), 1 mM ouabain (Sigma), 1 μg/ml antymycin A (Sigma), 5 μg/ml oligomycin (Calbiochem), 200 μM o-dianisidine (Sigma), and 1 mM parathion (Chem Service) in the same medium used for maintenance.

**Measurement of Mitochondrial Membrane Potential, pHi, ADP Import, ATP Concentration, NADH, and Caspase 3 Activity—**To measure Δψm, cells (1 × 10^6 cells/ml) deprived of cytokines for various times, treated with reagents (described above), or adjusted for pH were resuspended in 5 μg/ml solution of JC-1 (Molecular Probes) in phosphate-buffered saline (25). Because accumulation of the JC-1 dye is reversible, cells were maintained in a stable concentration of the dye throughout the time of measurement. After 20-min incubation at 37 °C, cells were immediately analyzed by flow cytometry. Dead cells were excluded by forward and side scatter gating. Data were accumulated by analyzing an average population of 20,000 cells. JC-1 aggregates were detectable in the fluorescein channel (emissions read at 527 nm), whereas JC-1 monomers were immediately analyzed by flow cytometry. Dead cells were also pretreated with 5 μg/ml oligomycin for 1 h before measuring mitochondrial membrane potential changes with JC-1. Where described under “Results,” cells were also pretreated with 1 μM nigericin for 1 h before measuring mitochondrial membrane potential changes with JC-1. Where described under “Results,” cells were also pretreated with 5 μg/ml oligomycin for 1 h before measuring mitochondrial membrane potential changes with JC-1.

**Manipulation of Intracellular pH—**To alter cytosolic pH in living, viable cells, FL5.12A cells were permeabilized to adjust intracellular pH to that of the extracellular buffers. Nigericin (1 μM) was used to permeabilize cells in a high K-Hepes buffer (25 mM Hepes, 145 mM KCl, 0.8 mM MgCl_2, 1.8 mM CaCl_2, 5.5 mM glucose) at pH 7.0, 7.5, or 8.0 for 30 min at 37 °C. Cells were then analyzed for mitochondrial inner membrane potential changes with JC-1. Where described under “Results,” cells were also pretreated with 5 μg/ml oligomycin for 1 h before measuring intracellular pH.

**Mitochondrial Protein Analysis—**Cell lysis was performed in isotonic buffer (pH 7.2), and the mitochondrial-enriched pellets were isolated as previously described for the ADP import assay (21). For detection of Bax and cytochrome c oxidase protein by Western blot, cell equivalent samples (10 μg protein from mitochodrial protein lysates (5 × 10^6 cells) were mechanically lysed and mitochondria were isolated in isotonic buffer (200 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Hepes, pH 7.2) (25). Mitochondria were incubated for 10 min at 37 °C, cells were immediately analyzed by flow cytometry. Dead cells were excluded by forward and side scatter gating. Data were accumulated by analyzing an average population of 20,000 cells. JC-1 aggregates were detectable in the propidium iodide channel (red fluorescence, emission at 590 nm), and JC-1 monomers were detectable in the fluorescein isothiocyanate channel (green fluorescence, emission at 527 nm) (26).

**To measure changes in intracellular pH, cells (1 × 10^6 cells/ml) deprived of cytokine for various times were treated with reagents (described above), or adjusted for pH were resuspended in 5 μg/ml solution of JC-1 (Molecular Probes) in phosphate-buffered saline (25). Because accumulation of the JC-1 dye is reversible, cells were maintained in a stable concentration of the dye throughout the time of measurement. After 20-min incubation at 37 °C, cells were immediately analyzed by flow cytometry. Dead cells were excluded by forward and side scatter gating. A pH calibration curve was generated by preloading cells with 1 μM BCECF-acetomethyl ester (Molecular Probes) followed by incubation for 30 min in a high K-Hepes buffer (25 mM Hepes, 145 mM KCl, 0.8 mM MgCl_2, 1.8 mM CaCl_2, 5.5 mM glucose) at pH 7.0, 7.5, or 8.0 for 30 min at 37 °C. Cells were then analyzed for mitochondrial inner membrane potential changes with JC-1. Where described under “Results,” cells were also pretreated with 5 μg/ml oligomycin for 1 h before measuring intracellular pH.

**RESULTS**

**Increase in Mitochondrial Membrane Potential Following Cytokine Withdrawal—**Respiring mitochondria generate a proton gradient across the inner membrane, producing a pH gradient and a membrane potential or Δψm. The Δψm represents most of the energy of the proton gradient, and using the fluorescent carbocyanine dye JC-1 (25, 30, 31), others have measured this potential. Lipophilic cations such as JC-1 accumulate in the mitochondrial matrix driven by the electrochemical gradient following the Nernst equation. The higher the Δψm (the more polarized the mitochondrial membrane), the more JC-1 dye is taken up into the matrix. In the cytosol, the monomeric form of the dye fluoresces green (emissions read at 527 nm), whereas highly concentrated within mitochondrial membrane, JC-1 forms aggregates that fluoresce red (emissions read at 590 nm) (32).

To quantitate the red fluorescence of cells that had taken up JC-1, we employed flow microfluorimetry throughout this study. Initially using fluorescent microscopy, we verified that this red fluorescence emanated from mitochondria (not shown). Thus, in a neutral pH solution, FL5.12A cells contained a few dots of red fluorescence against a diffuse greenish orange fluorescence; others have correlated this pattern to a Δψm of 140–160 mV (26). In an alkaline pH solution, we observed a dramatic increase in punctate red fluorescence (with no green fluorescence) reported to indicate a Δψm of greater than 190mV (26). Similar studies concluded that green fluorescing mitochondria were equivalent to a Δψm of less than 100 mV (26). Thus, a higher intensity of red fluorescence would indicate a higher Δψm (hyperpolarization), whereas loss of red and increased green fluorescence would indicate a reduced Δψm (dепolarization).

Using the pro-B cell line FL5.12A, which is dependent on IL-3 for survival, we examined early events following cytokine withdrawal. Deprivation of IL-3 resulted in death by 48 h with no cells surviving beyond 72 h. Fig. 1A, a two-dimensional display of JC-1 red fluorescence (y axis, 590 nm) versus green fluorescence (x axis, 527 nm), illustrates the quantitative changes in Δψm that occur following IL-3 withdrawal. At 0 h, in
IL-3 Withdrawal Increases Mitochondrial Membrane Potential

Fig. 1. Mitochondrial membrane potential detected using JC-1 fluorescence following IL-3 or IL-7 withdrawal. A, FL5.12A cells were incubated in medium lacking IL-3 for 0, 3, 5, 20, and 40 h or treated for 30 min with CCCP (50 µM) and assayed with JC-1 (5 µg/ml). In the top panels, at 0 h, peak red fluorescence was 30.8; at 3 h, peak red fluorescence was 77.4; and at 5 h, peak red fluorescence was 40.9. In the lower panels, by 20 h, depolarization of the mitochondrial inner membrane began (seen as increasing green fluorescent cells), maximizing at 40 h post-IL-3 withdrawal. Cells treated with CCCP underwent depolarization, confirming the capacity of the JC-1 probe to detect mitochondrial membrane potential changes. Shown is a representative example of six similar experiments. B, D1 cells were incubated in medium lacking IL-7 for 0, 4, or 6 h, then assayed with JC-1 (5 µg/ml). An increase in the red fluorescence emitted by the cell population was observed 4 h post-IL-7 withdrawal. Three similar experiments were performed. Quantification was determined by flow cytometry analysis, as described under “Experimental Procedures.” Lines defining quadrants were arbitrarily defined to highlight changes in the fluorescence of the cell populations. Percentages represent cell number in each quadrant. On the y axis, in log scale from 0.1 to 1000, red fluorescence (read in the propidium iodide channel, emissions at 590 nm) indicates the mitochondrial uptake of the JC-1 dye and the formation of J-aggregates within the matrix. On the x axis, in log scale from 0.1 to 1000, green fluorescence (read in the fluorescein isothiocyanate channel, emissions at 527 nm) indicates cytosolic retention of the JC-1 monomers. The uncoupling agent CCCP was included as a depolarization control to confirm the capacity of the JC-1 dye to measure $\Delta \psi_m$ changes and shows that depolarized cells appear in the lower right quadrant in this display (Fig. 1A). Following IL-3 withdrawal, depolarized cells (lower right quadrant) increased to 31.2% at 20 h and 61.5% at 40 h. The number of depolarized cells that maximized the number of dead cells at these time points (30–40% at 20 h and 60–70% at 40 h) IL-3 withdrawal, therefore, resulted first in an early, transient increase in $\Delta \psi_m$ (hyperpolarization) peaking at 3 h, followed later by depolarization that began at 20 h. The early hyperpolarization phase has not been described previously and was examined further in this study.

To verify that JC-1 accurately measured changes in $\Delta \psi_m$, we evaluated other fluorescent dyes. Shown in Table I is a representative experiment in which the dyes DiOC$_6$, Rhodamine 123, and JC-1 equally demonstrate their capacity for measuring changes in $\Delta \psi_m$ following IL-3 withdrawal. Numbers in Table I represent the peak fluorescence intensity at the optimal emission wavelength for each dye. All three dyes detected the increase in $\Delta \psi_m$ peaking 2–3 h after IL-3 withdrawal; therefore, the rise in $\Delta \psi_m$ was not an artifact of an individual dye. Because JC-1 allows dual emission detection for both the cytosolic monomeric form of the dye and the aggregated mitochondrial form of the dye, we favored it for subsequent analysis.

To determine whether alterations in $\Delta \psi_m$ were a general consequence of cytokine withdrawal, we evaluated changes in $\Delta \psi_m$ in an IL-7-dependent thymocyte cell line, D1. Shown in Fig. 1B, D1 cells exhibited a pattern similar to that seen in the FL5.12A cells. Hyperpolarization of $\Delta \psi_m$ observed as an increase in the red fluorescing cell population, was a transient and early event occurring 4–5 h after IL-7 withdrawal (Fig. 1B). In addition, mouse pro-T cells (CD3$^+$CD4$^+$CD8$^-$) responsive to IL-7 also showed increased $\Delta \psi_m$ following cytokine withdrawal. Therefore, the rise in $\Delta \psi_m$ we describe is not a phenomenon unique to the IL-3-dependent FL5.12A cell line.

Mitochondrial Hyperpolarization Is Independent of Caspases and the Bcl-2 Family of Proteins—We recently reported that following IL-3 or IL-7 withdrawal, intracellular alkalization triggered the translocation of the pro-apoptotic protein, Bax, to mitochondria (21). To address a potential role for Bax in the...
FMK, a caspase inhibitor, did not prevent the increase in histograms displaying the intensity of red fluorescence $\text{ZVAD-DiBac$_4$}$, a fluorescent probe used to measure plasma membrane potential (not shown). Therefore, the rise in potential, did not detect changes following cytokine withdrawal (data not shown) and caspase 3 activation (Fig. 2C). Hence, these anti-apoptotic proteins repress late apoptotic events, such as mitochondrial depolarization. Therefore, we conclude that translocation of Bax to the mitochondria is not required for the rise in $\Delta V_{m}$ induced by cytokine withdrawal.

We next addressed whether other apoptosis regulators, caspases, Bel-2, or Bel-X$_\text{L}$ could inhibit mitochondrial hyperpolarization during cytokine withdrawal. Shown in Fig. 2A (in histograms displaying the intensity of red fluorescence) ZVAD-FMK, a caspase inhibitor, did not prevent the increase in $\Delta V_{m}$, although it effectively blocked the later activation (24 h) of caspase 3 (Fig. 2C). Therefore, caspases do not participate at this early stage in the mitochondrial hyperpolarization process but are initially activated 20–24 h post-IL-3 withdrawal (Fig. 2C). Overexpression of the anti-apoptotic proteins Bel-2 or Bel-X$_\text{L}$ also did not interfere with the $\Delta V_{m}$ rise following IL-3 withdrawal (Fig. 2B). Hence, these anti-apoptotic proteins repress late apoptotic events, such as mitochondrial depolarization (data not shown) and caspase 3 activity (Fig. 2C), but not the transient increase in $\Delta V_{m}$.

Intracellular Alkalinization Induces Mitochondrial Hyperpolarization—The early increase in $\Delta V_{m}$ following cytokine (IL-3 or IL-7) withdrawal has not been reported previously; therefore, its time course was analyzed further using the FL5.12A cell line. In Fig. 3A, mitochondrial hyperpolarization (indicated by increased intensity of red fluorescence) peaked 2–3 h after IL-3 withdrawal. To relate JC-1 fluorescence to the actual mitochondrial membrane potential, other studies using JC-1 (26) reported a base-line range of 140–160 mV, which, if extrapolated linearly, would indicate that the hyperpolarization we observe is greater than 190 mV.

We next evaluated whether increased $\Delta V_{m}$ reflected a change in the plasma membrane potential. However, DiBac$_4$ was a fluorescent probe used to measure plasma membrane potential, did not detect changes following cytokine withdrawal greater than those observed by simply adding fresh medium (not shown). Therefore, the rise in $\Delta V_{m}$ following cytokine withdrawal does not correlate with an increase in the polarization of the plasma membrane.

Previously, we had examined the D1 and FL5.12A cells for other early intracellular events induced by IL-7 or IL-3 withdrawal and detected a rise in cytosolic pH (21), using the pH indicator BCECF (33). As shown in Fig. 3B, this rise in intracellular pH measured in the FL5.12A cells followed a parallel time course to that of the increase in $\Delta V_{m}$ seen in Fig. 3A. We also reported a similar time course of alkalization following IL-7 withdrawal from D1 cells, with a peak pH rise occurring 4–6 h post-withdrawal (21), paralleling the rise in $\Delta V_{m}$ (Fig. 1B).

Because these two early, transient events, the increase in $\Delta V_{m}$ and intracellular alkalization, occurred together following IL-3 or IL-7 withdrawal, we tested whether alkaline pH induced mitochondrial hyperpolarization. Using nigericin (a K$^+$/H$^+$ ionophore) and a high K$^+$ buffer (145 mM KCl) to equilibrate intracellular pH to that of the extracellular buffer, we adjusted the intracellular pH of FL5.12A cells to 7.0, 7.5, or 8.0. This method effectively fixes pH (as confirmed by measurement of intracellular pH; data not shown) in the presence or absence of IL-3. It should be noted that nigericin dissipates the pH gradient of membranes and can cause a slight hyperpolarization in response to the flux of K$^+$ ions. However, in our manipulation of pH with nigericin, this effect is negligible as is demonstrated in further control experiments. As shown in Fig. 4A (upper panels), elevating intracellular pH to 7.5 or 8.0 induced a rise in $\Delta V_{m}$ comparable with that seen during IL-3 withdrawal. No further increase in $\Delta V_{m}$ occurred beyond pH 8, possibly because of the effect of nigericin on plasma membrane permeability, which at higher doses limits the concentration of cytosolic JC-1 monomers (data not shown).

To confirm that the JC-1 dye was in fact measuring increased $\Delta V_{m}$ versus directly responding to pH, we assayed the effect of pH on JC-1 in a cell-free solution. Using JC-1 (300 nM) in a buffer containing mannitol, sucrose, succinate, and Mg$^+$-ATP (31), the effect of pH 6.5–9.0 on fluorescence was measured by fluorometry. The red fluorescence of JC-1 (emissions read at 590 nm) was decreased at acidic pH, was maximal at neutral pH, and did not increase further at alkaline pH. The green fluorescence of JC-1 was not pH-sensitive. Thus, alkaline pH does not directly increase the red fluorescence emission of JC-1 but rather increases the uptake of JC-1 into mitochondrial matrix in response to a heightened $\Delta V_{m}$.

An Oligomycin-sensitive Mechanism Causes Mitochondrial Hyperpolarization in Response to Alkalization: Implication of the $F_0F_1$-ATPase—To determine how alkaline pH induced the rise in $\Delta V_{m}$, we evaluated a possible role for the $F_0F_1$-ATPase, a protein complex spanning the inner mitochondrial membrane, oligomycin, a specific inhibitor of $F_0F_1$-ATPase, completely prevented the increase in red fluorescence or hyperpolarization induced by pH 7.5 or 8.0. This implicated the $F_0F_1$-ATPase in the mode of reverse proton gradient of membranes and can cause a slight hyperpolarization.

Having proven that oligomycin prevented alkaline-pH-induced hyperpolarization, the results displayed in Fig. 4C indicated that oligomycin was also effective in inhibiting the rise in $\Delta V_{m}$ following IL-3 withdrawal. In the presence of IL-3 (i.e., normal cells), oligomycin produced a slight hyperpolarization, a consequence of inhibiting the dissipation of the proton gradient.

| IL-3 withdrawal | Mean peak fluorescence |
|------------------|------------------------|
| JC-1 dye (590 nm) | DiOC$_6$ dye (525 nm) | Rhodamine 123 dye (525 nm) |
| h                |                        |                        |
| 0                | 13.1 4.56              | 1.15                   |
| 1                | 28.2 4.71              | 1.35                   |
| 2                | 31.2 4.88              | 2.00                   |
| 3                | 34.3 5.88              | 2.70                   |

| Mean peak fluorescence | IL-3 withdrawal |
|------------------------|------------------|
| JC-1 dye (590 nm)      |                  |
| DiOC$_6$ dye (525 nm)  |                  |
| Rhodamine 123 dye (525 nm) |          |

Comparison of dyes used for the detection of changes in the mitochondrial membrane potential.

3. A. R. Khaled, K. Kim, D. K. Ferris, K. Muegge, A. N. Moor, L. Fliegel, and S. K. Durum, submitted for publication.
and ATP synthesis by the F$_{0}$F$_{1}$-ATPase (Fig. 4C). Note that this increase in $\Delta \Psi_m$ caused by oligomycin was not equal in magnitude to that induced by either alkaline pH (Fig. 4A) or cytokine withdrawal (Fig. 4C). Thus, the increased $\Delta \Psi_m$ we describe is not solely due to the inhibition of ATP synthesis but requires an additional active process, likely the F$_{0}$F$_{1}$-ATPase pumping protons in reverse across the inner mitochondrial membrane.

The effect of oligomycin on $\Delta \Psi_m$ is distinct from that of compounds that directly inhibit the electron transport chain (the generator of the proton gradient) and in this manner deplete ATP levels. For example, treatment with antimycin A (which blocks electron transport) caused a rapid loss of $\Delta \Psi_m$ or depolarization (shown by the decrease in red fluorescence) similar to that caused by valinomycin, which induces mitochondrial swelling and depolarization (Fig. 4C), and the uncoupler, CCCP (Fig. 1A).

The ionophore, nigericin, when added during IL-3 withdrawal, also had no appreciable effect on $\Delta \Psi_m$ (Fig. 4B) as was shown by others (30), nor did incubation in high K$^+$ buffers

![Fig. 2](http://www.jbc.org/Downloaded from http://www.jbc.org/) and ATP synthesis by the F$_{0}$F$_{1}$-ATPase (Fig. 4C). Note that this increase in $\Delta \Psi_m$ caused by oligomycin was not equal in magnitude to that induced by either alkaline pH (Fig. 4A) or cytokine withdrawal (Fig. 4C). Thus, the increased $\Delta \Psi_m$ we describe is not solely due to the inhibition of ATP synthesis but requires an additional active process, likely the F$_{0}$F$_{1}$-ATPase pumping protons in reverse across the inner mitochondrial membrane.

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(145 mM KCl) used to adjust intracellular pH (data not shown). Because nigericin dissipates the pH gradient but induces an ion flux across membranes, it can cause a slight hyperpolarization when added to healthy cells. However, this was only observed at higher concentrations of nigericin (1 μM; data not shown) and not at the 1 mM range we used in our experiments (Fig. 4C). This suggests that the effect of nigericin on the mitochondrial pH gradient would be minor in comparison with the impact high intracellular alkalinity would have following cytokine withdrawal.

Alkalization Inhibits ADP Transport into Mitochondria—The increase in ΔΨm was inhibited by oligomycin, suggesting that it was caused by the F0F1-ATPase working in reverse, hydrolyzing ATP and pumping protons outward across the mitochondrial membrane. However, several conditions under which the F0F1-ATPase was known to reverse, for example low ΔΨm or oxygen deprivation, were not observed during IL-3 withdrawal or alkalization. The concentrations of mitochondrial ADP and ATP can affect the direction of F0F1-ATPase function. Therefore, we examined the mitochondrial levels of ADP in response to pH. Shown in Table II, is an experiment in which mitochondrial protein lysates were extracted from cells whose intracellular pH was fixed with nigericin and high K+ buffer (145 mM KCl) at pH 6.5, 7.2, and 8.1 for 15–20 min.
Alkaline pH induced a steep decline in mitochondrial ATP and a modest rise in mitochondrial ADP. To explain this, we next measured the effect of pH on the import of ADP into isolated mitochondria. At pH 8.1, [3H]ADP import decreased 5-fold compared with pH 6.5 and 4-fold compared with pH 7.2 (Fig. 5A). Thus, the extreme depletion of mitochondrial ADP could explain the reversal of the F$_{0}$F$_{1}$-ATPase, inducing hyperpolarization. To evaluate whether the alkalinization effect could be mimicked by blocking the ADP/ATP exchanger ANT, cells were treated with ZVAD-FMK, an inhibitor of a high affinity ADP binding site on ANT, and assayed for changes in $\Delta \Psi_m$. After 1–4 h of treatment with CAT (dose range from 50 $\mu$M to 1 mM), no increases in $\Delta \Psi_m$ were observed; in fact, a decrease in membrane polarization occurred, possibly a result of CAT inducing PT pore formation (data not shown). Thus, although both CAT and alkalinity block ADP import, the pH effect on membrane polarization is not mimicked by CAT for unknown reasons. The block in ADP transport induced by alkalinity could be due to alterations in the charge of adenosine nucleotides, affecting their transport, or could result from changes in mitochondrial protein conformation and function (i.e. VDAC channel activity). Nevertheless, by whatever means alkaline pH inhibits ADP transport, the intramitochondrial deficit in ADP may induce the reversal of the F$_{0}$F$_{1}$-ATPase.

A Transient Decrease in ATP and Accumulation of Pyridine Nucleotides Follows Cytokine Withdrawal—Mitochondria are the major source of cellular ATP. From the preceding findings, following cytokine withdrawal, mitochondrial production of ATP should decline if the substrate, ADP, were reduced and if the major ATP producer, F$_{0}$F$_{1}$-ATPase, were hydrolyzing rather than synthesizing ATP (17). Total intracellular ATP did decline sharply as shown in Fig. 5A. From 1 to 3 h after IL-3 withdrawal, there was a large decrease (4–5-fold) in ATP concentration; this decrease coincided with the period of mitochondrial hyperpolarization (Fig. 3A) and intramitochondrial alkalinization (Fig. 3B).

To determine how much of the ATP decline was due to alkaline pH, we tested the effect of adjusting intracellular pH (in the presence of IL-3) using the nigericin and high K$^+$ buffer (145 mM KCl) method. This verified that alkaline intracellular pH could induce ATP depletion as shown in Fig. 5B. Note that the ATP depletion induced by alkalization is less severe than that induced by IL-3 withdrawal alone (Fig. 5A); this difference could be attributed to the length of incubation time used to adjust intracellular pH with nigericin (30 min) versus withdrawal IL-3 withdrawal (3 h). The longer incubation time would permit the effects of ADP transport inhibition to produce a larger decline in total ATP levels. Ouabain was also tested (data not shown) because it inhibits the plasma membrane K$^+$/Na$^+$-ATPase, which can be a major consumer of ATP, but it failed to restore ATP levels in either IL-3-withdrawal or pH-induced conditions. Treatment with oligomycin during cytokine withdrawal could only restore the ATP levels up to 20% of normal (data not shown); this suggests that most of the ATP decrease could result from failure to synthesize ATP (because of lack of ADP as a substrate), in addition to the actual consumption of ATP by the F$_{0}$F$_{1}$-ATPase.

Hyperpolarization of the $\Delta \Psi_m$ did not result in the immediate generation of ROS as measured by the levels of superoxide (O$_{2}^{-}$). We observed that during the first 4 h of IL-3 withdrawal, the levels of O$_{2}^{-}$ were only slightly lower than what was measured in the presence of IL-3 and did not increase until 20–24 h after withdrawal (data not shown). Furthermore, treatment with the antioxidant N-acetyl cysteine had no protective effect from death following cytokine withdrawal (data not shown).

Hyperpolarization of the $\Delta \Psi_m$ however, could potentially interrupt mitochondrial respiration. To evaluate this, we measured the levels of NADH, an early substrate used in the electron-transport process, coupled to oxidative phosphorylation, to synthesize ATP. The level of NADH in cells (mainly from within mitochondria) can be determined by blue autofluorescence (450 nm). Fig. 6A shows that IL-3 withdrawal resulted in accumulation of NADH at a time shortly following the peak of alkalinization (Fig. 3B) and the rise in $\Delta \Psi_m$ (Fig. 3A). NADH accumulation was also produced by treatments with oligomycin (inhibitor of the F$_{0}$F$_{1}$-ATPase) or antimycin A (electron transport blocker) (Fig. 6B). In contrast, valinomycin (which swells and depolarizes mitochondria) did not increase NADH levels (Fig. 6B) and in fact led to a slight reduction. Therefore, alkaline pH altered mitochondrial chemistry, producing an accumulation of reduced pyridine nucleotides, oxidation of which would have been the critical first step of electron transport.

Parathion Induces Mitochondrial Hyperpolarization and Cell Death Independent of Bax, Bcl-2, or Caspases—We then evaluated whether an increase in $\Delta \Psi_m$ could induce cell death, and if so, whether the pro-apoptotic protein, Bax, was required for cell death. The organophosphorus compound, parathion, can partition into mitochondrial membranes and has previously been shown to induce mitochondrial hyperpolarization in human neuroblastoma cells (34). As shown in Fig. 7A, parathion induced hyperpolarization of the mitochondrial inner membrane in P15.12A cells after 2 h in the presence of IL-3. Like the increase in $\Delta \Psi_m$ produced during cytokine withdrawal, this hyperpolarization was independent of caspases (because ZVAD-FMK had no effect), Bax (because a Bax$^{-/-}$ cell line hyperpolarized), and Bcl-2 (because cell lines expressing Bcl-2 hyperpolarized) (Fig. 7A).

Because the mitochondrial translocation of Bax is an early event induced by cytokine withdrawal (21), we tested whether mitochondrial hyperpolarization could “attract” or “repel” Bax. Parathion, which induced hyperpolarization, did not inhibit Bax translocation to the mitochondria during IL-3 withdrawal and, in fact, induced Bax translocation in the presence of IL-3 (Fig. 7B). Although this might suggest that Bax is attracted by hyperpolarized mitochondria, other experimental approaches (not shown) suggest that the two are unrelated; moreover, Bax is thought to insert into the outer membrane, whereas the above effects are on the inner membrane. After 24 h of treatment, parathion, in the presence of IL-3, induced mitochondrial depolarization (data not shown), caspase 3 activation (Fig. 7C), and cell death (Fig. 7D).

Parathion-mediated cell death was not attributable to a mechanism involving Bax or Bcl-2, based on its effect on cells deficient in the former or overexpressing the latter (Fig. 7D). Bcl-2 did, however, protect from caspase 3 activation (Fig. 7C) as it also did following IL-3 withdrawal (Fig. 2C). Therefore, the results using parathion suggest that mitochondrial hyperpolarization could lead to cell death, although it is also quite possible that parathion has toxic effects in addition to induction of hyperpolarization.

**DISCUSSION**

In this study, we show that withdrawal of cytokines from dependent cell lines induces several early interconnected pro-
cesses. Within 2–3 h after IL-3 withdrawal, intracellular pH rose to over pH 7.8. This alkalinization inhibited mitochondrial ADP import, which could cause a reversal of the F0F1-ATPase, inducing hyperpolarization of the inner mitochondrial membrane, decrease of ATP, and the oxidation of NADH. None of these early events involved the Bcl-2 family of proteins or caspases. These phenomena were not unique to IL-3, because similar responses (alkalinization and hyperpolarization) occurred in IL-7-dependent cells. We suggest that these events represent significant stresses to the cell following cytokine withdrawal, rendering the mitochondria and other cellular components vulnerable to damage leading to cell death.

Changes in mitochondrial membrane integrity immediately precede apoptotic death (35). Some studies have described disruption of the mitochondrial membrane concurrent with cytochrome c release and formation of the PT pore. The sequence of these late events include expansion of the matrix, rupture of the outer membrane, and the release of caspase-activating proteins (36). A previous study using rhodamine 123 concluded that a form of mitochondrial hyperpolarization shortly pre-
IL-3 Withdrawal Increases Mitochondrial Membrane Potential

A.

Parathion induces mitochondrial hyperpolarization, Bax translocation, caspase 3 activation, and cell death independent of the Bcl-2 family of proteins. A, FL5.12 cells, expressing the empty neomycin vector (Vector) or Bcl-2 cells cultured with IL-3, and the immortalized wild-type or Bax−/− cell line were incubated with or without 1 μM parathion for 2 h. FL5.12 (Vector) cells were also treated with 100 μM ZVAD-FMK. Cells were then stained with JC-1 and assayed by flow cytometry. Parathion induced mitochondrial hyperpolarization. Peak positions of red fluorescence for cell populations are displayed. B, FL5.12 cells were treated with or without parathion and IL-3 for 3 h. Mitochondrial protein lysates were made as described under “Experimental Procedures,” and levels of Bax protein were assayed by Western blot. Cytochrome c oxidase is included as a control for the quality of the mitochondrial lysates. Both IL-3 withdrawal and parathion treatment induced the mitochondrial translocation of Bax. C and D, FL5.12 cells, expressing the empty neomycin vector (vector), or Bcl-2 grown in IL-3, and the immortalized wild-type or Bax−/− cell line were cultured with or without 1 mM parathion for 2 h, and levels of caspase 3 activity or cell viability (by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue assay) were assayed 20 h later as previously described (15). Expression of Bcl-2, but not the lack of Bax, protected cells from caspase 3 activation but not from cell death. All results are shown in duplicate. Error bars represent standard error of the mean.

In our study, using JC-1 (and other dyes), although confirming that mitochondria depolarize just prior to their disruption (Fig. 1) and the activation of caspases (Fig. 2C), we now describe a very early increase in ΔΨm (2–3 h after cytokine withdrawal) that does not coincide with the generation of ROS (which occurs 20–24 later) and is not solely produced by the loss of proton gradient dissipation (Fig. 4C). Furthermore, overexpression of Bcl-2 or Bcl-XL does not block this earlier increase in ΔΨm (Fig. 2A). In a recent report, stimulation of the CD95/Fas/Apo-1 receptor, inducing apoptotic death in lymphocytes, resulted in the elevation of ΔΨm within 20 min to 1 h of receptor engagement and preceded the later cell death, as measured by phosphatidylserine externalization, by several hours (38). Similarly, staurosporine treatment of Jurkat T cells induced an increase in ΔΨm within 20 min, preceded cytochrome c release, and was followed by a decrease in ATP (39). Thus, induction of cell death, either by cytokine withdrawal, the activation of a death receptor, Fas, or staurosporine treatment is preceded by an early and transient rise in the ΔΨm. We previously observed that staurosporine treatment induced intracellular alkalinization, suggesting that this could be a common mechanism inducing hyperpolarization.

In addition to Bcl-2 and Bcl-XL, protecting mitochondria from damage (12, 22), the pro-apoptotic protein Bax can induce formation of the PT pore and cytochrome c release (11), perhaps through Bax associating with VDAC and ANT (40–42). However, in our experiments, we found no requirement for Bax in the early hyperpolarization of the ΔΨm (Fig. 7), nor was it observed in p53-induced apoptosis (37). Hence, the Bcl-2 family of proteins can regulate the later disruption of the mitochondria (depolarization) and activation of caspase 3 (Fig. 2C), whereas the early intracellular changes described herein (alkalinization and hyperpolarization) do not appear to result from the activity of Bax or the loss of Bcl-2 or Bcl-XL.

The mitochondrial hyperpolarization and ATP depletion that we observed following IL-3 withdrawal could be due to high intracellular pH blocking the import of ADP, in turn causing the mitochondrial F0F1-ATPase to reverse. The F0F1-ATPase is a protein complex comprised of two reversible rotary motors (43). The F1 subunit can normally synthesize ATP or, when reversed, hydrolyze ATP. An additional level of control exists in that a natural inhibitor protein, IF1, which is pH-sensitive, also regulates the F0F1-ATPase. IF1 can prevent the unneeded hydrolysis of ATP, as could occur in the absence of oxygen when glycolysis becomes the main source of ATP (17, 44). Although IF1 may partly account for the hypothesized effect of pH on F0F1-ATPase, the more important control could be the limited availability of ADP, ADP import (and ATP export) is primarily through the VDAC/ANT complex. In a recent study, growth factor withdrawal caused the VDAC/ANT complex to stop ATP/ADP exchange after 12–18 h of IL-3 withdrawal (23). Loss of outer membrane permeability to anions was later implicated in VDAC closure (45). That is distinguished from the process we describe here, in that the deficiency in ATP/ADP exchange shown in those studies was inhibited by Bcl-XL (which also...
prevented disruptions in the $\Delta \Psi_{m}$ in their studies) (23), whereas ours is not (Figs. 2 and 7). Perhaps the striking inhibitory effect of alkaline pH on ADP transport is due to changes in the conformation or charge distribution of mitochondrial proteins or substrates, altering these in such a way that is affected by Bel-2 family members.

As a result the elevated electrochemical gradient or $\Delta \Psi_{m}$ interruption of electron transport and oxidative phosphorylation may result. If the proton gradient is not dissipated by ATP synthesis and a steeper proton concentration gradient results, NADH oxidation could cease (16). It would require increased energy to move more protons across the membrane in the face of the heightened proton-motive force. Likewise, mitochondria containing NADH, $O_{2}$, and $P_i$ but no ADP could no longer accumulate of NADH after IL-3 withdrawal (as shown in Fig. 6A).

How do these early events during cytokine withdrawal relate to the late events of apoptosis, such as mitochondrial disruption and caspase activation? To address this, we induced mitochondrial hyperpolarization with the organophosphorus compound, parathion (34). Parathion induced cell death, with mitochondrial hyperpolarization $per se$ could lead to cell death, independent of the apoptotic cascade (Fig. 7).

In conclusion, we show that long before the morphological changes of apoptosis occur, cells deprived of their trophic factors have compromised mitochondrial function. Although these processes were transient and reversible (cells could still be rescued by readmission of the cytokine), this disruption of essential cellular activities could contribute in time to the sequelae ending in cell death.

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REFERENCES

1. Blalock, W. L., Weinstein-Oppenheimer, C., Chang, F., Hoyle, P. E., Wang, X. Y., Algate, P. A., Franklin, R. A., Oberhaus, S. M., Steelman, L. S., and McCullorey, J. A. (1999) Leukemia 13, 1109–1166
2. Johnson, D. E. (1998) Prost. Biosci. 3, 4333–4354
3. Kinoshita, T., Yokota, T., Arai, K., and Miyajima, A. (1995) Oncogene 10, 2207–2212
4. Rinaudo, M. S., Su, K., Falk, L. A., Halder, S., and Mufson, R. A. (1995) J. Biol. Chem. 270, 30–38
5. del-Peso, L., Gonzalez, G. M., Page, C., Herrera, R., and Nunez, G. (1997) Science 278, 687–689
6. Liu, X., Kim, C. N., Yang, J., Emmerson, R., and Wang, X. (1996) Cell 86, 147–157
7. Klück, R. M., Bosso-Wetzell, E., Green, D. R., and Newmeyer, D. D. (1997) Science 275, 1102–1106
8. Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J. L., Petit, P. X., and Kroemer, G. (1995) J. Exp. Med. 181, 1661–1672
9. Zamzami, N., Susin, S. A., Marchetti, P., Hirsch, T., Gomez-Monterrey, I., Castedo, M., and Kroemer, G. (1996) J. Exp. Med. 183, 1533–1544
10. Marchetti, P., Castedo, M., Susin, S. A., Zamzami, N., Hirsch, T., Macho, A., Haefner, A., Hirsch, F., Geuskens, M., and Kroemer, G. (1996) J. Exp. Med. 184, 1155–1160
11. Nair, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R. J., Matsuda, H., and Tsujimoto, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14681–14686
12. Rosse, T., Olivier, R., Monnay, L., Rager, M., Conus, S., Fcellay, L., Jansen, B., and Borrner, C. (1998) Nature 391, 496–499
13. Minamikawa, T., Williams, D. A., Bowser, D. N., and Nagley, P. (1999) Exp. Cell Res. 246, 26–37
14. Krohn, A., Wachlicki, T., and Prehn, J. H. (1999) J. Neurosci. 19, 3294–7401
15. Finucane, D. M., Waterhouse, N. J., Amarante-Mendes, G. P., Cotter, T. G., and Green, D. R. (1999) Exp. Cell Res. 251, 166–174
16. Aichert, B., Bray, D., Lewin, A., and Jan, B. M. (1998) Molecular Biology of the Cell, 3rd Ed., Garland Publishing Inc, New York
17. Guerrieri, F., Scarfo, R., Zamzami, F., Che, Y. W., and Papa, S. (1987) FEBS Lett. 213, 67–72
18. Harris, D. A., and Das, A. M. (1991) Biochem. J. 280, 561–573
19. Papa, S., Zamfoti, F., Cozzo, C., Perrucci, C., Candita, C., and Minuto, M. (1996) Eur. J. Biochem. 240, 461–467
20. Ross, W., and Broe, C. W. (1989) J. Biol. Chem. 264, 15224–15229
21. Khaled, A. R., Kim, K., Hofmeister, R., Muegge, K., and Durum, S. K. (1999) Proc. Natl. Acad. Sci. U. S. A. 90, 11476–11481
22. Vander, H. M., Chandel, N. S., Schumacker, P. T., and Thompson, C. B. (1997) Cell 91, 627–637
23. Vander, H. M., Chandel, N. S., Schumacker, P. T., and Thompson, C. B. (1999) Mol. Cell 3, 159–167
24. Lichtman, A. M., Reynolds, D. S., Fallar, D. Y., and Abbas, A. K. (1986) Nature 324, 489–491
25. Salvioli, S., Ardizzone, C., Franceschi, C., and Cossarizza, A. (1997) FEBS Lett. 411, 77–82
26. Reers, M., Smiley, S. T., Mottola-Hartshorn, C., Chen, A., Lin, M., and Chen, L. B. (1995) Methods Enzymol. 260, 406–417
27. Franck, P., Petitpain, N., Cheri, M., Dardennes, M., Maachi, F., Schutz, B., Puissen, L., and Nabet, P. (1996) J. Biotechnol. 46, 187–195
28. Theorell, B. (1983) Cytometry 4, 61–65
29. Bender, J. G., Van, E., van, J. M., and Steinkamp, J. A. (1995) J. Leukocyte Biol. 58, 603–611
30. Cossarizza, A., Barcarani-Contri, M., Kalashnikova, G., and Franceschi, C. (1993) Biochem. Biophys. Res. Commun. 197, 49–55
31. Reers, M., Smiley, S. T., and Chen, L. B. (1991) Biochemistry 30, 4480–4486
32. Smiley, S. T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T. W., Steele, G. D. J., and Chen, L. B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3671–3675
33. Weinlich, M., Theis, C., Lin, C. T., and Kinne, R. K. (1996) J. Exp. Biol. 201, 57–62
34. Carlson, C., and Ehrich, M. (1999) Toxicol. Appl. Pharmacol. 160, 33–42
35. Susin, S. A., Zamzami, N., and Kroemer, G. (1996) Biochem. Biophys. Acta 1356, 151–165
36. Green, D. R., and Reed, J. C. (1998) Science 281, 1309–1312
37. Li, P.F., Dietz, R., and von Harsdorf, R. (1999) EMBO J. 18, 6027–6036
38. Banki, K., Hutter, G., Eichenroth, N. J., and Perl, A. (1999) J. Immunol. 162, 1466–1479
39. Scarlett, J. L., Sheard, P. W., Hugues, G., Ledgerwood, E. C., Ku, H-H., and Lebowitz, M. S., and Pedersen, P. L. (1996) Arch. Biochem. Biophys. 330, 342–354
40. Vander, H. M., Chandel, N. S., Li, H., Schumacker, P. T., Colombini, M., and Thompson, C. B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4666–4671
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