An Early Oxygen-Dependent Step Is Required for Dexamethasone-Induced Apoptosis of Immature Mouse Thymocytes[1]

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The roles of oxygen and reactive oxygen intermediates in apoptosis are unclear at present. Although oxygen and reactive oxygen intermediates are not required for the execution of apoptosis, oxygen may be involved in at least some forms of apoptosis. In this study we show that dexamethasone (Dex)-induced apoptosis of immature mouse thymocytes is completely inhibited by hypoxic culture. In contrast, anti-CD95 thymocyte apoptosis is unaffected by hypoxia, indicating the existence of two forms of thymocyte apoptosis: an oxygen-dependent pathway (Dex induced) and an oxygen-independent pathway (anti-CD95 induced). Furthermore, hypoxia inhibited mitochondrial permeability transition (PT) in Dex-treated, but not in anti-CD95-treated, thymocytes, suggesting that the oxygen-sensitive step is upstream of mitochondria. Both Dex- and anti-CD95-induced PT and apoptosis were dependent on activation of IL-converting enzyme-like protease, as PT and apoptosis were inhibited by preincubation with Cbz-Val-Ala-Asp-fluoromethyl ketone, an irreversible inhibitor of IL-converting enzyme-like proteases. In addition, hypoxia inhibited the activation by Dex of caspase-3 (CPP32)-like proteases. Our data show that the private signaling pathways of Dex (oxygen dependent) and anti-CD95 (oxygen independent) both converge upstream of mitochondrial changes. The oxygen-dependent step in Dex-induced apoptosis lies upstream of caspase-3-like protease activation. Our observations support a model of apoptosis signaling in which independent pathways (oxygen dependent and oxygen independent) particular to each stimuli converge at a central point in the apoptotic cascade. ***The Journal of Immunology,*** **2000, 165: 4822–4830.**

Apoptosis is a morphologically distinct form of cell death that is characterized by DNA condensation and fragmentation, early exposure of phosphatidylserine, and cell shrinkage (1, 2). Although the morphologic appearance of apoptotic cells is well described, the signaling pathway or pathways leading to it are not yet fully understood. Recently, oxidative stress was proposed as a central mediator of apoptosis (3). This hypothesis stemmed from several observations that linked oxidative stress to apoptosis. These observations include the induction of oxidative stress by apoptotic stimuli such as TNF-α (4, 5) and gamma and UV irradiation (6); apoptosis inhibition by antioxidants such as *N*-acetylcysteine (7, 8), catalase (8), spermine (9), and 3,3,5,5-tetramethylpyrroline *N*-oxide (TMPO)[3] (10); and the direct induction of apoptosis by hydrogen peroxide (H_2O_2) (11). However, this concept suffered a major setback when it was shown that oxygen was not required for the execution of some forms of apoptosis (12, 13). This was concluded after observing that apoptosis induced by staurosporine, anti-CD95/Fas Abs or IL-3 withdrawal was not prevented by culture under very low oxygen conditions (0.002% O_2) (12). More recently, however, and in contrast to these observations McLaughlin et al. showed that apoptosis after glucocorticoid, PMA/ionomycin, or staphylococcal enterotoxin B stimulation was inhibited in the absence of oxygen (14), leading to the possibility that some forms of apoptosis are oxygen dependent. However the point at which oxygen may be acting within the apoptotic pathway is unknown.

Kroemer et al. have recently proposed a signaling model for apoptosis in which several private pathways (receptor ligation, DNA-damaging agents, ionizing radiation, etc.) converge in the mitochondria, inducing a permeability transition (PT) of its membrane and a drop in mitochondrial membrane potential (2). This group has argued that this biochemical event could act as a central regulator of apoptosis, coordinating different death signals and their private pathways into one common effector pathway. Since redox can regulate PT (15), it is possible that reactive oxygen species are involved in apoptosis by mediating PT (16). However, the importance of mitochondrial PT has been challenged by recent findings that release of cytochrome *c* from the mitochondria, a step that seems to be required for the activation of the IL-converting enzyme (ICE) protease cascade, can occur without a drop in the mitochondrial membrane potential (17, 18), proving that death can occur without PT. These observations have raised doubts about the significance of this biochemical event. However, if PT is important for apoptosis, determining PT in oxygen-dependent and -independent apoptosis may reveal the existence of early distinct O_2-regulated pathways that are particular to stimuli that induce O_2-dependent apoptosis.

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[3] Abbreviations used in this paper: TMPO, 3,3,5,5-tetramethylpyrroline *N*-oxide; PT, permeability transition; Dex, dexamethasone; NDGA, nordihydroguaiaretic acid; NMMA, N^G-monomethyl-L-arginine; $DiOC_6$3, 3,3′-dihexyloxacarbocyanine iodide; Ac-DEVD-AMC, (*N*-acetyl)-DEVD-7 amino-4-methylcoumarin; z-VAD-fmk, Cbz-Val-Ala-Asp-fluoromethyl ketone; z-FA-fmk, Cbz-Phe-Ala-fluoromethyl ketone; $\Delta\Psi_m$, mitochondrial transmembrane potential; ICE, IL-converting enzyme.

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In this paper we show that in immature mouse thymocytes glucocorticoid-induced cell death is inhibited under hypoxic conditions. In contrast, anti-CD95-induced apoptosis of thymocytes is not prevented under low oxygen conditions, indicating that thymocytes can respond to some apoptotic stimuli in hypoxia and that oxygen is not required for the execution of the final apoptotic program. Furthermore, we show that both glucocorticoid induced mitochondrial PT and caspase-3-like protease activation were inhibited under low oxygen conditions, suggesting that the oxygen-dependent step is upstream of these events. Rotenone, an inhibitor of mitochondrial complex I, and TMPO, a nitrone spin trap, both inhibited apoptosis and PT in glucocorticoid-treated thymocytes, suggesting that inhibition of oxidant generation is the mechanism for the inhibitory effect of hypoxia. Finally, activation of the caspase protease cascade is required for both glucocorticoid and anti-CD95 apoptotic pathways, since all evidence of apoptosis, including mitochondrial alterations, is prevented by ICE-like protease inhibitors. These findings taken together indicate that during glucocorticoid-induced apoptosis both the induction of mitochondrial PT and caspase-3-like protease activation occur after the oxygen-dependent step. Our findings clearly show that some apoptotic stimuli induce O_2-dependent apoptosis and that this O_2 requirement is an early event in the apoptosis signaling cascade.

Materials and Methods

Reagents

Dexamethasone (Dex), rotenone, TMPO, desferioxamine, malonate, nordihydroguaiaretic acid (NDGA), and indomethacin were all purchased from Sigma (St. Louis, MO). N^G-monomethyl-L-arginine (NMMA) was obtained from Calbiochem (La Jolla, CA). Hoechst 33342 and 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$3) were purchased from Molecular Probes (Eugene, OR). Annexin V was a gift from Jonathan Tait (University of Washington, Seattle, WA). Anti-CD95 mAbs and (*N*-acetyl)-DEVD-7 amino-4-methylcoumarin (Ac-DEVD-AMC) were obtained from PharMingen (San Diego, CA). RPMI 1640 (Life Technologies, Gaithersburg, MD) and FCS (Life Technologies) contained <0.03 and 0.3 endotoxin units/ml endotoxin, respectively.

Culture conditions

Thymocytes obtained from male BALB/CN mice (4–6 wk old) were washed once in RPMI 1640 medium, passed through a nylon mesh, and resuspended in complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 20 mM HEPES, and 10 U/ml penicillin/streptomycin). Thymocytes were cultured at 10^6/ml in 24-well plates for either 2 or 5 h at 37°C in aerobic (20% O_2 and 5% CO_2) or hypoxic conditions (90.0% N_2, 5% CO_2, and 5% H_2) in the presence or the absence of Dex (1 μM)- and anti-CD95 Ab-coated latex beads or isotype control Ab-coated beads. Latex beads (Interfacial Dynamics, Portland, OR; 10^7/ml) were coated with anti-CD95 or isotype control mAb (PharMingen) at 5 μg/ml in PBS for 2 h at 37°C. Coated beads were washed twice with PBS, resuspended in complete medium, and incubated for 30 min at 37°C. Beads were centrifuged and resuspended in complete medium. Coated beads were mixed with thymocytes at a 1:1 ratio. For protein synthesis inhibition experiments thymocytes were preincubated with 1 or 10 μg/ml cycloheximide (Sigma) for 2 h before the addition of Dex- or anti-CD95-coated beads.

Hypoxic cultures were performed in an integrated hypoxic hood and incubator (Bactron Anaerobic Chambers, Sheldon Mfg., Cornelius, OR) in an atmosphere of 90% N_2, 5% CO_2, and 5% H_2. Oxygen levels were constantly monitored with an oxygen sensor (Cole Palmer, Chicago, IL). Oxygen was maintained below 0.02% for all experiments. Before introducing any reagents into the hypoxic hood, they were deoxygenated at least six times by extracting air and exchanging it with a mixture of 90% N_2, 5% CO_2, and 5% H_2 in a pressurized pass chamber. Complete culture medium and 24-well polystyrene plates were allowed to equilibrate in the hypoxic hood for at least 24 h before experiments. Thymocytes were pelleted, introduced in the hypoxic hood, and resuspended in the hypoxic medium.

To inhibit superoxide generation thymocytes were treated with rotenone (500 nM), an inhibitor of mitochondrial respiratory complex I. At the same time thymocytes were treated with either Dex- or anti-CD95-coated latex beads or were left untreated. TMPO, a nitrone-based spin trap (10), was used at 40 mM. Malonate (5 mM), an inhibitor of mitochondrial respiratory complex II, was used as an inhibitor of oxidative metabolism. NDGA, an inhibitor of lipoxygenase, was used at 50 μM. Indomethacin, an inhibitor of cyclo-oxygenase, was tested at 100 μM. NMMA, an inhibitor of NO synthase, was used at 100 μM. The above reagents were titrated at the following concentrations: TMPO, 1, 10, 20, 40, and 100 mM; malonate, 5 and 10 mM; NDGA, 10, 30, 50, 100, 250, and 500 μM; indomethacin, 10, 50, 100, and 200 μM; and NMMA, 10, 50, 100, and 200 μM. The above reagents were used in the apoptosis inhibition experiments at the highest concentration that did not enhance spontaneous thymocyte death.

To inhibit ICE-like proteases, thymocytes were preincubated for 4 h with either 50 μM Cbz-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk; Enzyme Systems Products, Dublin, CA), an irreversible inhibitor of ICE-like proteases; 50 μM Cbz-Phe-Ala-fluoromethyl ketone (z-FA-fmk; Enzyme Systems Products, Dublin, CA), a dipeptide, as a negative control; or medium alone before treatment with Dex-, anti-CD95 Ab-, or isotype control Ab-coated beads. Thymocytes were cultured for either 2 or 5 h after preincubation at 37°C in aerobic conditions.

Quantitation of death, mitochondrial permeability, and surface staining

Thymocytes were harvested and resuspended in staining medium (biotin/phenol red-deficient RPMI 1640, 3% FCS, and 0.05% NaN_3). Death was quantified by staining with either 1 μg/ml Hoechst 33342 (19) or 25 nM annexin V-FITC in staining medium for 20 min on ice. Mitochondrial PT was assessed by measuring mitochondrial transmembrane potential ($\Delta\Psi_m$). Thymocyte $\Delta\Psi_m$ was measured using 80 nM $DiOC_6$3 for 15 min at 37°C (20, 21). In some experiments thymocytes were also stained with surface markers anti-CD4-PE (PharMingen) and anti-CD8-Cy5-PE (PharMingen) for 20 min on ice. Cells were washed three times and immediately analyzed in a FACStar Cell Sorter (Becton Dickinson, Mountain View, CA) at the Stanford Shared FACS Facility. Data analysis was performed using Desk software (22). Cell counting was performed using a Coulter counter (Coulter, Hialeah, FL). Absolute numbers of live and dead cells were calculated by multiplying the percentage of Hoechst-negative (live) and Hoechst-positive (dead) cells by the total number of cells in the culture. Results are presented as the mean ± SE of three independent experiments.

DNA extraction and gel electrophoresis

Thymocytes (1.5×10^6) were washed once and resuspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% sarcosyl, and 0.5 mg/ml of proteinase K) and incubated for 1 h at 50°C. After addition of 5 μg of RNase, each sample was incubated for another hour at 50°C. DNA was electrophoresed in a 0.75% agarose gel at 100 V for 1 h. Gel was stained with ethidium bromide and visualized under UV light.

RNA isolation and RT-PCR

Total RNA was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD) as recommended by the manufacturer. Briefly, after 3-h treatment with Dex, thymocytes (3×10^7 cells) were harvested and resuspended in 3 ml of TRIzol and incubated for 5 min at room temperature. After adding 0.6 ml of chloroform, samples were centrifuged at 12,000 × *g* for 15 min at 4°C. The aqueous phase was recovered, and RNA was precipitated with isopropanol (0.5 ml/1 ml of TRIzol used). The RNA pellet was washed with ice-cold 70% ethanol once and resuspended in diethylpyrocarbonate-treated water (23). First-strand cDNA synthesis was performed using the Life Technologies Superscript first strand synthesis system. In brief, 1 μg of RNA was incubated with 200 ng of random hexamer primers in the presence of 500 nM dNTP, 1.25 mM $MgCl_2$, 10 mM DTT, 40 U of RNaseOUT (Life Technologies) recombinant ribonuclease inhibitor, and 50 U of Superscript II RT in 1× RT buffer (20 mM Tris-HCl (pH 8.4) and 50 mM KCl). Reactions were incubated at 25°C for 10 min, then at 42°C for 50 min, and were terminated at 70°C for 15 min. PCR was performed on 2 μl of cDNA in 1× PCR buffer (20 mM Tris-HCl (pH 8.4) and 50 mM KCl), 200 μM dNTP, 2 mM $MgCl_2$, 1 μM of each primer, and 1 U of Taq DNA polymerase (Life Technologies). The final volume was 25 μl. Samples were denatured at 94°C for 1 min and then were incubated at 94°C for 40 s, at 61°C for 40 s, and at 72°C for 40 s for 22 cycles (*GILZ*) or 16 cycles (β-actin). Primer pairs used for *GILZ* were 5′-GAA CAC CGA AAT GTA TCA GAC-3′ and 5′-GGG GCT TGC CAG CGT CTT CAG-3′ (expected PCR product, 309 bases); those used for β-actin were 5′-TGG GTC AGA AGG ACT CCT ATG-3′ and 5′-ACC AGA CAG CAC TGT GTT GGC-3′ (expected PCR product, 765 bases). PCR products were electrophoresed at 75 V for 1 h in a 1% agarose gel in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) buffer. Gels were stained

with ethidium bromide and then visualized and quantitated under UV using the Gel Doc 2000 system (Bio-Rad, Hercules, CA).

Caspase-3-like protease activation

Caspase-3-like protease activation following Dex treatment was measured in thymocytes cultured under normal or hypoxic conditions. Caspase-3-like protease activation was measured in cellular extracts with a protease assay that uses Ac-DEVD-AMC (PharMingen) as a substrate (24). Briefly, 2×10^6 cells were lysed in cell lysis buffer (10 mM HEPES/KOH (pH 7.4), 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 5 mM DTT, 1 mM PMSF, 10 μg/ml pepstatin A, 20 μg/ml leupeptin, and 10 μg/ml aprotinin). Cell extracts were then centrifuged at 14,000 rpm for 10 min, supernatants were transferred to new tubes, and protein concentration was measured by the Bradford assay (Bio-Rad). The protease assay was performed in a 96-well plate (Maxisorb, Nunc, Copenhagen, Denmark). Thirty microliters of protein extract was assayed in 200 μl of protease assay buffer: 20 μM Ac-DEVD-AMC, 20 mM HEPES (pH 7.5), 10% glycerol, and 2 mM DTT. Plates were incubated at 37°C for 0.5, 1, 1.5, 2, and 2.5 h and then read at 460 nm with a Victor multicolor reader (Wallac, Turku, Finland).

Results

Hypoxia inhibits Dex-induced, but not anti-CD95-induced, apoptosis of immature mouse thymocytes

To determine whether redox chemistry played a role in Dex-induced apoptosis in immature mouse thymocytes, we treated freshly isolated thymocytes with Dex under both aerobic (20% O_2) and anaerobic (<0.02% O_2) conditions. As shown in Fig. 1*A*, Dex, a synthetic glucocorticoid, was an efficient inducer of apoptosis. At 5 h, 47.1 ± 2.9% (mean ± SE of three independent experiments; $n = 3$) of thymocytes had undergone apoptosis after treatment with Dex in aerobic conditions. However, Dex was unable to signal apoptosis in the absence of oxygen (28.3 ± 2.9% apoptosis in untreated vs 28.4 ± 2.7% in Dex-treated cultures; $n = 3$). Hypoxia also inhibited DNA fragmentation induced by Dex treatment (Fig. 1B) and enhanced the survival of thymocytes in Dex-treated cultures (data not shown). Following 5-h culture, absolute cell numbers did not differ between untreated and Dex- or anti-CD95-treated cultures (data not shown). Hypoxia increased the spontaneous death of thymocytes in 5-h cultures (28.3 ± 2.9% spontaneous apoptosis in hypoxia vs 18.3 ± 3.0% spontaneous apoptosis in aerobic cultures; $n = 3$; Fig. 1*A*). In contrast to Dex, anti-CD95-induced apoptosis of thymocytes was unaffected by hypoxic culture. Anti-CD95 was capable of inducing death in both aerobic (40.6 ± 5.5% anti-CD95 treated vs 23.9 ± 0.4% isotype control treated; $n = 3$) and hypoxic conditions (54.1 ± 8.5% anti-CD95 treated vs 30.8 ± 2.1% isotype control treated; $n = 3$; data not shown and Fig. 2). Hypoxia-induced spontaneous thymocyte death did not exhibit DNA fragmentation (data not shown). Apoptosis measured using annexin V staining and flow cytometry showed similar results (data not shown). Inhibition of protein synthesis by cycloheximide resulted in inhibition of Dex-induced apoptosis and enhanced CD95-induced apoptosis of thymocytes (Fig. 3). Therefore, the inhibition of Dex-induced apoptosis under hypoxic conditions was not the result of a hypoxia-mediated general inhibition of protein synthesis, as Fas-induced thymocyte apoptosis was not enhanced under hypoxia. Therefore, thymocytes are capable of responding to some apoptotic stimuli under low oxygen conditions, confirming that oxygen is not required for the execution of apoptosis in thymocytes.

It was possible that the protection rendered by hypoxia in Dex-treated thymocytes was due to an inhibition of glucocorticoid signaling rather than the apoptotic cascade. Therefore, to approach this question, we studied the transcription of *GILZ*, a gene in mouse thymocytes that is significantly up-regulated by glucocorticoids (25). Using RT-PCR, we found that Dex treatment for 3 h induces the up-regulation of *GILZ* approximately 3- to 4-fold, and

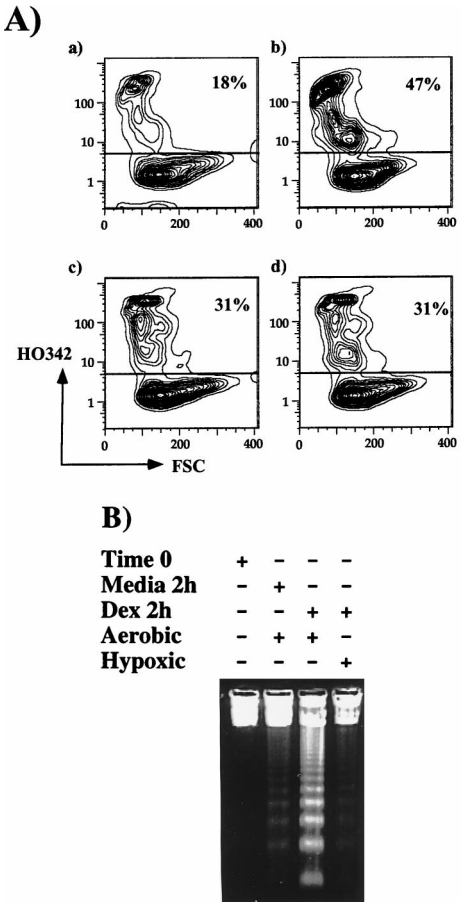

FIGURE 1. Dex-induced apoptosis of immature mouse thymocytes is inhibited in hypoxia. *A*, Thymocytes from BALB/CN (4- to 6-wk-old) male mice were cultured either unstimulated (*a* and *c*) or stimulated with Dex (1 μM) under aerobic (*a* and *b*) or hypoxic (*c* and *d*) conditions for 5 h at 37C. Apoptosis was quantified by staining thymocytes with Hoechst 33342. The percentage of apoptotic cells is shown in the *upper right corner*. A representative experiment is shown of at least three independent experiments performed. *B*, Dex-induced apoptosis DNA fragmentation of mouse thymocytes is inhibited under hypoxic conditions. Cells were cultured for 5 h in the presence or the absence of 1 μM Dex under aerobic or hypoxic conditions. FSC, Forward light scatter.

that the level of *GILZ* up-regulation by Dex was similar in aerobic and hypoxic cultures, thus confirming that glucocorticoids can signal under hypoxia (Fig. 4).

The above findings show that Dex is unable to induce apoptosis under hypoxia, suggesting at least two forms of apoptosis: an oxygen-dependent pathway (Dex-induced death) and an oxygen-independent pathway (anti-CD95-induced apoptosis).

Hypoxia inhibits mitochondrial permeability transition in Dex-induced apoptosis, but not in anti-CD95-induced apoptosis

Mitochondria have been proposed to play a central role in the regulation of apoptosis by coordinating different apoptotic signals into one common effector pathway. The mitochondrial PT has been proposed as the biochemical event indicating mitochondrial involvement in apoptosis (2). Although disruption of mitochondrial membrane potential ($\Delta\Psi_m$) is an indirect correlate of apoptosis, its central role in apoptosis and its early kinetics (26) permit study of the temporal sequence of early events during apoptosis signaling. We examined the effect of aerobic and hypoxic conditions on PT. Fig. 2 shows PT in both Dex- and anti-CD95-induced

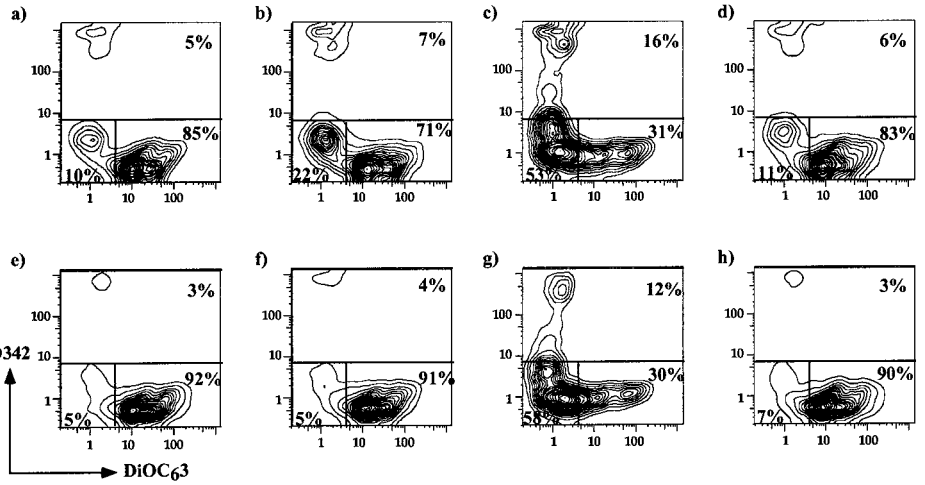

FIGURE 2. Hypoxia inhibits mitochondrial PT and apoptosis in Dex-treated, but not anti-CD95-treated, thymocytes. Thymocytes either unstimulated (*a* and *e*) or stimulated with Dex (*b* and *f*), anti-CD95-coated latex beads (*c* and *g*), or isotype-coated beads (*d* and *h*) were cultured for 2 h at 37°C in either aerobic (*a–d*) or hypoxic (*e–h*) conditions. Mitochondrial PT was measured by staining $DiOC_6 3$. Apoptosis was quantified by staining with Hoechst 33342. The percentage of cells undergoing PT is shown in the *lower left corner*. A representative experiment is shown of three independent experiments performed.

apoptosis. After 2 h of aerobic culture 23.1 ± 1.1% of DEX-treated cells and 47.9 ± 5.5% of anti-CD95-treated cells ($n = 3$) had decreased retention of $DiOC_6 3$, indicating loss of their mitochondrial membrane potential ($\Delta\Psi_m$) in aerobic conditions. In contrast, only 11.2 ± 1.8% of cells in untreated cultures had decreased $DiOC_6 3$ retention. In both forms of apoptosis, thymocytes underwent PT before demonstrating other changes associated with apoptosis, including exposure of phosphatidylserine (measured by annexin V), DNA condensation and plasma membrane integrity loss (measured by Hoechst 33432 and 7-actinomycin D), drop in reduced glutathione (measured by monochlorobimane), and scatter changes (drop in forward scatter and increase in side scatter; data not shown). Previous work suggested that superoxide anion generation occurred after the permeability transition (27), raising the possibility that the protection rendered by hypoxia occurred after the mitochondrial changes had occurred. To test this possibility, retention of $DiOC_6 3$, was measured after DEX and anti-CD95 treatment in aerobic and hypoxic culture conditions. Culture in hypoxia completely inhibited permeability transition in Dex-treated cells while not affecting anti-CD95-treated thymocytes (Fig. 2). After 2 h of culture, 23.2 ± 1.1% of Dex-treated cells in aerobic conditions had decreased retention of $DiOC_6 3$ (background in aerobic cultures, 11.8 ± 1.8%; $n = 3$), while only 4.3 ± 0.8% of Dex-treated cells in hypoxia had undergone PT (background level in hypoxia, 4.3 ± 0.5%), suggesting that the hypoxic checkpoint was upstream of the mitochondrial changes. PT induced by anti-CD95 treatment was unaffected by low oxygen conditions, as similar proportions of cells underwent PT after anti-CD95 stimulation under aerobic and hypoxic conditions (47.9 ± 5.5 vs 46.1 ± 11.5%, respectively; Fig. 2).

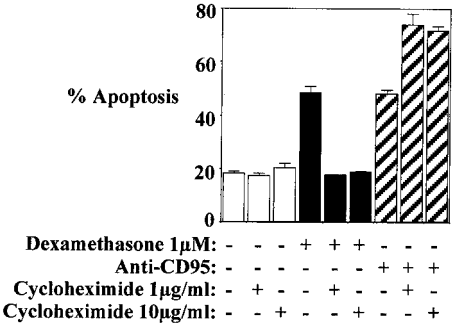

FIGURE 3. Protein synthesis inhibition abrogates Dex-induced thymocyte apoptosis and enhances CD95-induced thymocyte apoptosis. Thymocytes were preincubated with 1 or 10 μg/ml cycloheximide (CHX) for 2 h before treatment with Dex- or anti-CD95-coated latex beads. Cells were incubated for 5 h, and apoptosis was quantified using Hoechst 33342. A representative experiment is shown of two independent experiments performed. □, Untreated cells; ■, Dex-treated cells; ▨, anti-CD95-treated cells.

Rotenone, a complex I mitochondrial inhibitor, and TMPO, a nitrone spin trap, inhibit Dex-induced, but not anti-CD95-induced, PT and apoptosis

Since not only oxidant generation but also oxidative metabolism are inhibited in the absence of oxygen (28), it was possible that

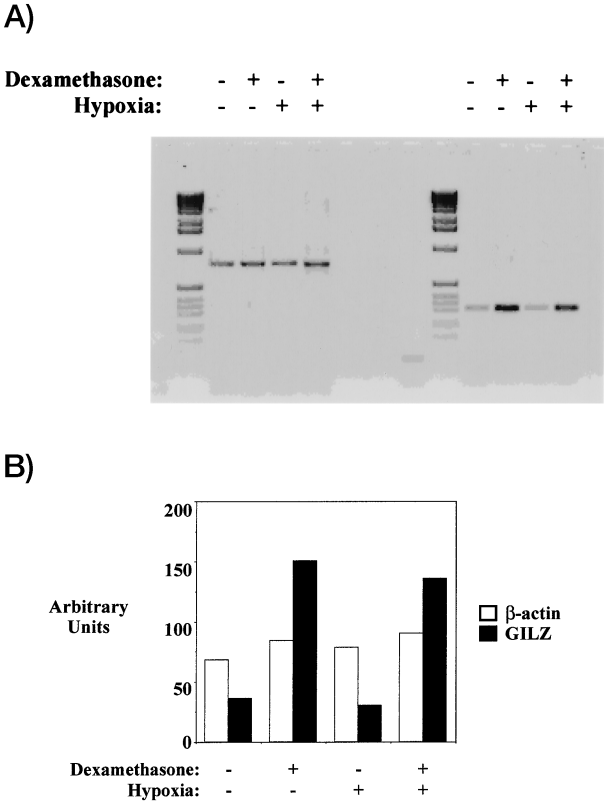

FIGURE 4. The Dex-induced transcription is not inhibited by hypoxia. *A*, *GILZ* transcription is induced by 3-h Dex treatment of thymocytes. *B*, Arbitrary density units shown for the above gel. A representative experiment of three performed is shown.

Table I. *Effect of inhibitors of metabolism, cytoplasmic oxidant generating sites, and electron spin trap on Dex-induced apoptosis*

Inhibitor	Site of Inhibition	% Thymocyte Apoptosis			
		Media	Media + inhibitor	Dex	Dex + inhibitor
Rotenone	Complex I	14.5 ± 2.3[a]	11.7 ± 0.8	53.4 ± 3.1	21.7 ± 2.1
Malonate	Complex II	12.7 ± 1.4	13.4 ± 3.1	50.0 ± 4.2	45.3 ± 3.7
NMMA	NO synthase	17.4 ± 4.8	21 ± 7.6	46.2 ± 3.6	45.0 ± 7.4
NDGA	Lipoxygenase	13 ± 0.3	45 ± 7.7	48.9 ± 3.4	36.8 ± 1.3
Indomethacin	Cyclooxygenase	12.7 ± 2.1	18.6 ± 2.3	49.8 ± 4.2	48.9 ± 2.1
TMPO	Spin trap	19.7 ± 1.1	20.3 ± 1.2	61.8 ± 0.7	22.3 ± 1.2

[a] Mean ± SE.

hypoxia was exerting its effect by inhibiting metabolism. To examine this possibility we tested the effects of several inhibitors of the mitochondrial respiratory chain and their effects on Dex-induced apoptosis in aerobic conditions. Rotenone, an inhibitor of complex I of the mitochondrial respiratory chain, partially inhibited Dex-induced apoptosis after 5 h of culture (53.4 ± 3.1% Dex treated vs 21.7 ± 2.0% Dex plus rotenone treated; Table I), while not affecting anti-CD95-induced apoptosis (data not shown). However, malonate, a complex II inhibitor, did not block Dex-induced apoptosis after 5 h of culture (Table I). These observations suggested that rotenone's effect was due to the inhibition of oxidant generation at mitochondrial complex I, rather than to a general inhibition of metabolism. To further explore this possibility we examined the effect of TMPO, a nitrone spin trap, in Dex-induced apoptosis. After 5 h of culture, TMPO partially inhibited Dex-induced apoptosis (61.8 ± 0.7% apoptosis in Dex-treated thymocytes vs 22.3 ± 1.2% apoptosis in Dex- plus TMPO-treated cultures; $n = 3$; Table I), suggesting that oxidant generation was required for Dex-induced death. Since the production of mitochondrial oxidants might be involved in Dex-induced apoptosis of thymocytes, we explored whether some of the other intracytoplasmic oxidant production sites were involved. After 5 h of culture, inhibitors of lipoxygenase (NDGA), cyclo-oxygenase (indomethacin), and NO synthase (NMMA) did not block Dex-induced apoptosis, suggesting that the required oxidants are produced by mitochondria (Table I). In addition, desferioxamine, an iron chelator, did not block Dex-induced apoptosis (data not shown).

To explore whether the rotenone and TMPO effects were kinetically situated at the same level as hypoxia, we tested the effects of these inhibitors on mitochondrial PT after 2 h of culture. Rotenone completely inhibited PT in Dex-treated thymocytes after 2 h of culture (22.5 ± 0.5% Dex treated vs 7.2 ± 0.8% rotenone plus Dex treated; $n = 3$; Fig. 5). As with hypoxia, rotenone decreased the spontaneous background from 9.5 ± 0.5 to 5.4 ± 0.6% of cells. TMPO also inhibited Dex-induced PT (22.6 ± 0.5 vs 12.5 ± 3.4% for Dex alone and Dex plus TMPO, respectively; Fig. 5), suggesting inhibition of oxidants rather than lack of oxidative metabolism as the explanation for the protective effect of hypoxia on both Dex-induced apoptosis and PT. Neither rotenone nor TMPO inhibited anti-CD95-induced PT or apoptosis (data not shown). Taken together, these data suggest that the inhibitory effect of hypoxia is due to the inhibition of mitochondrial oxidant generation.

Inhibition of ICE-like proteases blocks PT and apoptosis in both DEX- and anti-CD95-treated thymocytes

To determine whether anti-CD95-induced and Dex-induced PT were regulated by ICE protease activation, we preincubated thymocytes for 4 h in the presence of z-VAD-fmk, an irreversible inhibitor of ICE-like proteases; medium alone; or z-FA-fmk, a dipeptide negative control. Two hours after adding the apoptotic stimuli, Dex and anti-CD95 treatments induced mitochondrial PT in 18.0 ± 2.2 and 48.6 ± 6.3%, respectively (background, 6.3 ± 0.7%), of thymocytes pretreated in culture medium alone (Fig. 6). At this time point there was no difference in the amount of dead cells (19.1 ± 3.4% in medium alone, 21.3 ± 5.2% in Dex treated, and 22.1 ± 3.6% in anti-CD95 treated). In contrast, Dex- and anti-CD95-induced PT was inhibited in z-VAD-fmk-pretreated thymocytes; 9.6 ± 1.6 and 14.5 ± 7.5% of z-VAD-fmk-pretreated

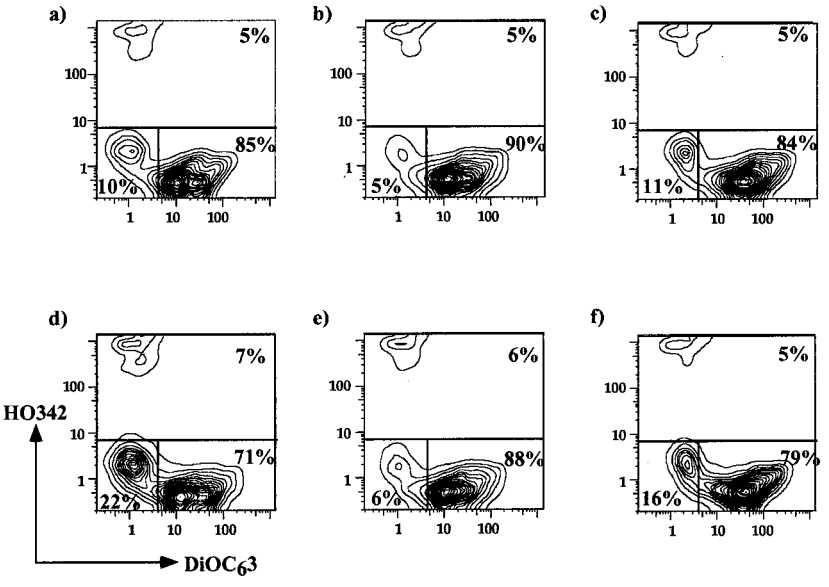

FIGURE 5. Rotenone and TMPO inhibit PT in Dex-induced apoptosis. Thymocytes were cultured for 2 h at 37°C in aerobic conditions in medium alone (*a*), medium plus rotenone (*b*), medium plus 40 mM TMPO (*c*), 1 μM Dex (*d*), 1 μM Dex plus 500 nM rotenone (*e*), or 1 μM Dex plus 40 mM TMPO (*f*). Mitochondrial PT was measured using $DiOC_6 3$. Apoptosis was quantified by staining with Hoechst 33342. The percentage of cells undergoing PT is shown in the *lower left corner*. A representative experiment is shown of three independent experiments performed.

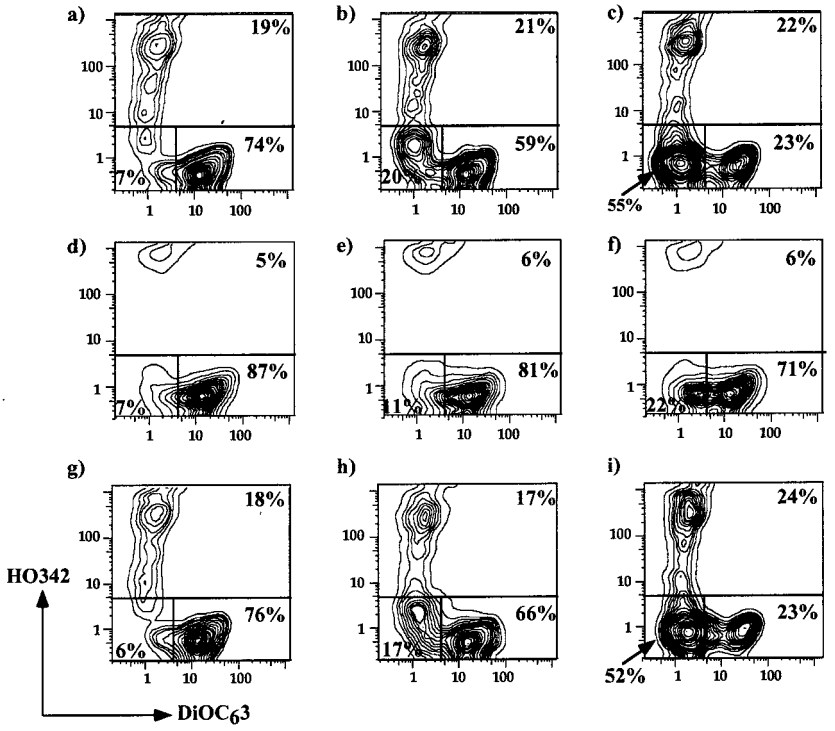

FIGURE 6. z-VAD-fmk, an irreversible inhibitor of ICE-like proteases, blocks PT in both Dex- and anti-CD95-induced apoptosis. Thymocytes were preincubated for 4 h at 37°C in aerobic conditions in medium alone (*a–c*), 50 μM z-VAD-fmk (*d–f*), or z-FA-fmk, a dipeptide negative control (*g–i*). After preincubation, cells were either left unstimulated (*a*, *d*, and *g*) or were stimulated with 1 μM Dex (*b*, *e*, and *h*) or anti-CD95-coated latex beads (*c*, *f*, and *i*) for 2 h at 37°C under aerobic conditions. Mitochondrial PT was measured using $DiOC_6 3$. Apoptosis was quantified by staining with Hoechst 33342. The percentage of cells undergoing PT is shown in the *lower left corner*. A representative experiment is shown of three independent experiments performed.

thymocytes stimulated for 2 h with Dex and anti-CD95, respectively, had undergone PT, indicating that mitochondrial changes were ICE dependent and downstream of ICE-like protease activation (Fig. 6). The inhibition was specific, as the thymocytes pretreated with z-FA-fmk showed no inhibition of Dex- and anti-CD95-induced PT. z-VAD-fmk pretreatment also inhibited apoptosis and DNA fragmentation in both Dex- and anti-CD95-treated thymocytes after 5 h of culture (data not shown and Fig. 7). These findings suggest that ICE-like proteases are involved in the Dex- and anti-CD95-induced apoptotic cascade upstream of the mitochondria.

To further explore whether the effect of hypoxia was upstream of caspase activation, we measured caspase-3-like activity in protein extracts prepared from Dex-treated thymocytes under aerobic and hypoxic conditions. Caspase-3-like activity was completely inhibited by hypoxia in both 2- and 5-h Dex-treated thymocytes (Fig. 8). Untreated thymocytes showed no caspase-3-like activity under either aerobic or hypoxic cultures.

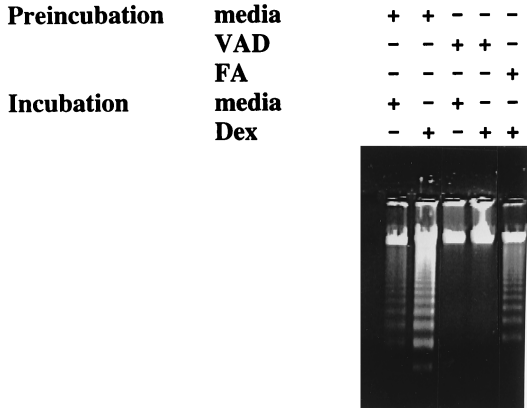

FIGURE 7. z-VAD-fmk inhibits DNA fragmentation in Dex-treated thymocytes. Thymocytes were preincubated for 4 h at 37°C in aerobic conditions in medium alone, 50 μM z-VAD-fmk, or z-FA-fmk, a dipeptide negative control. After preincubation, cells were cultured for 5 h at 37°C in either the presence or the absence of 1 μM Dex under aerobic conditions. A representative experiment is shown of two independent experiments performed.

Discussion

In this study we have provided evidence for the existence of an early oxygen-sensitive pathway in the induction of Dex-induced apoptosis in immature mouse thymocytes. We showed that Dex-treated thymocytes cultured in hypoxic conditions do not undergo typical apoptotic changes such as early mitochondrial permeability transition, phosphatidylserine surface exposure, cell shrinkage, DNA condensation, and fragmentation. This effect was not due to the cell's inability to respond to apoptotic stimuli in hypoxia, as anti-CD95-induced thymocyte apoptosis was unaffected by hypoxia, or to inhibition of the glucocorticoid signaling pathway, as the up-regulation of transcription of *GILZ*, a glucocorticoid-regulated gene, was not inhibited. Our observations suggest that inhibition of oxidant generation might be responsible for the inhibitory

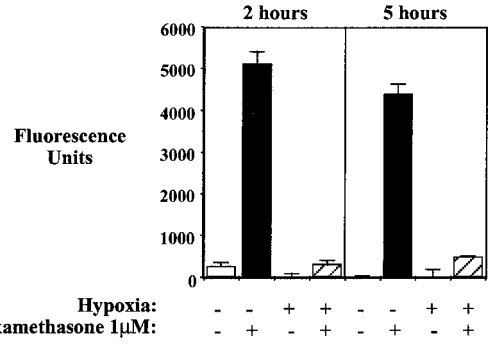

FIGURE 8. Dex-induced caspase-3-like activity in thymocytes is inhibited by hypoxia. Thymocytes treated for 2 and 5 h are shown. A representative experiment of two experiments performed is shown.

effect of hypoxia, since rotenone, an inhibitor of mitochondrial complex I that blocks the production of O_2^-, and TMPO, a nitrone spin trap that can neutralize O_2^-, both blocked Dex-induced apoptosis. Since O_2^- can lead through Fenton chemistry to the production of hydroxyl radicals ($^{\cdot}OH$) in the presence of iron, it was possible that $^{\cdot}OH$ and not O_2^- was involved in glucocorticoid-induced apoptosis. However, we excluded this possibility by showing that iron chelators did not affect Dex-induced apoptosis.

The above findings show that O_2^- generated at the mitochondria is involved in Dex-induced apoptosis. The production of such O_2^- at the mitochondria could be important in opening redox-sensitive mitochondrial pores by directly oxidizing glutathione (15, 16). The opening of these pores could lead to mitochondrial PT and the release of cytochrome *c* and/or apoptosis-inducing factor from mitochondria into the cytoplasm, two mitochondrial proteins that have been implicated in apoptosis (17, 18, 29, 30). However, such a direct induction of PT by O_2^- during Dex-induced apoptosis would not be consistent with our observations. ICE inhibitors blocked PT and apoptosis in both Dex- and anti-CD95-treated thymocytes, showing that ICE activation precedes PT. Since caspase-3-like protease activity was inhibited by hypoxia, it is possible that the O_2-dependent step during Dex-induced apoptosis lies upstream of caspase activation in general. Caspase-3-like activity is observed within 90 min of Dex treatment of thymocytes (31), and this is in agreement with our findings. Although these findings suggest that caspase-3 activity is an early event, the exact temporal relationship between caspase-3-like protease activation and mitochondrial PT during Dex-induced thymocyte apoptosis has not yet been established. Therefore, our data support a role for O_2 in the Dex signaling cascade, which is independent of mitochondrial PT and may be placed at a level before both PT and caspase-3-like protease activation.

Our observations can be explained in the model proposed by Kroemer et al. in which several private pathways converge at a central effector pathway (2). The two independent pathways distinguished by hypoxic culture (oxygen dependent and oxygen independent) could represent the private pathways of Dex (oxygen dependent) and Fas (oxygen independent), respectively. The dependence of both these pathways on activation of the ICE proteolytic cascade suggests that they converge at this point. Our data show that this activation of ICE-like proteases is upstream of mitochondrial PT for both Dex- and Fas-induced apoptosis, since mitochondrial PT can be inhibited by z-VAD-fmk in both. Recently, it was shown that activation of ICE family proteases during Fas- and ceramide-induced apoptosis occurs both upstream and downstream of mitochondrial PT (24). These complex interactions most likely will also be the case for Dex-induced apoptosis. Our data, however, show that the oxygen-dependent step in Dex-induced apoptosis is upstream of PT and caspase-3-like protease activation. At present we cannot exclude that other members of the caspase family may be acting upstream of this O_2-dependent step; our data, however, clearly show that this step is at least upstream of caspase-3-like proteases and raises the possibility the O_2-dependent step is upstream of caspase activation in general. There is evidence that Dex-induced apoptosis is not the only oxygen-dependent pathway. McLaughlin et al. reported that superantigen- and PMA/ionomycin-induced apoptosis are both inhibited in low oxygen conditions (14). In preliminary experiments we also found that activation-induced cell death of human PBMC is inhibited in hypoxia (J. F. Torres-Roca, D. R. Greenwald, and P. D. Katsikis, unpublished observations). These observations should explain why antioxidants can affect some, but not all, forms of apoptosis. Oxygen, therefore, is required by some stimuli in the early signaling events before ICE activation, but it is clearly not required for the effector phase of apoptosis.

We found that hypoxia induced spontaneous thymocyte death in 5-h cultures. This raises the question of whether hypoxia alone can induce apoptosis in thymocytes. The nature of this hypoxic death is not clear at present; however, it does not appear to be apoptotic, as no DNA fragmentation or caspase-3-like activity was observed in these cells. Whether this death is necrosis induced by hypoxia or an effect of reoxygenation during harvesting of these cells remains to be determined. In addition, the spontaneous thymocyte death induced by hypoxia may be interfering with Dex-induced apoptosis under hypoxia. Our data suggest that this is not the case, since 2-h hypoxia alone induced no mitochondrial PT, while mitochondrial PT induced by 2-h Dex treatment was completely inhibited by hypoxia. Furthermore, Fas-induced apoptosis still proceeded in the presence of hypoxic death. These observations, we believe, make it unlikely that the hypoxia-induced spontaneous death interferes with Dex-induced apoptosis under hypoxia. However, we cannot exclude at present that a stress or adaptive response to hypoxia of thymocytes undergoing a slow nonapoptotic death may be playing a role in the inhibition of Dex-induced, but not CD95-induced, apoptosis. Spontaneous thymocyte death under aerobic conditions, on the other hand, exhibited DNA fragmentation and could be inhibited with the ICE inhibitor z-VAD-fmk, suggesting that it is apoptotic in nature. It should be noted, however, that caspase-3-like activity could not be demonstrated in these cells.

Hypoxia may be inhibiting Dex-induced apoptosis by inhibiting protein synthesis or even inducing anti-apoptotic proteins, such as Bcl-2, and thus inhibiting Dex-induced apoptosis by an indirect mechanism. Recent studies have indicated that protein synthesis inhibition in neurons can up-regulate Bcl-2 and antioxidant pathways, resulting in protection from oxidative insults (32). For such a neuroprotective effect to occur, however, cells had to be preincubated with protein synthesis inhibitors. It is unlikely that protein synthesis is inhibited substantially in our 5-h experiments, since CD95-induced thymocyte apoptosis is not enhanced in hypoxia, and we have shown here that cycloheximide treatment of thymocytes enhances CD95-induced apoptosis significantly. The possibility still remains, however, that hypoxia inhibits protein synthesis specifically in Dex-treated thymocytes, but not in CD95-treated cells. Whether hypoxia up-regulates Bcl-2 is not known at present, but such Bcl-2 up-regulation would inhibit Dex-induced, but not Fas-induced, thymocyte apoptosis (33, 34) and would be consistent with our findings. Although we cannot exclude such a mechanism being involved in the inhibitory effect of hypoxia on Dex-induced apoptosis, our findings, showing inhibition of Dex-induced mitochondrial PT at 2 h, would require that Bcl-2 up-regulation occurs by 2 h of hypoxia. Given that in our experiments Dex is added immediately after thymocytes are placed in hypoxia, such an up-regulation of Bcl-2 must be very rapid.

Cytochrome *c* release from the mitochondria and ATP are both required for the formation of the Apaf-1/caspase 9 complex, which, in turn, initiates a caspase cascade during apoptosis (35). This is particularly important for our studies, as Apaf-1 and caspase 9 are both required for Dex-induced, but not CD95-induced, thymocyte apoptosis (36–38). Furthermore, ATP depletion inhibits Dex-induced thymocyte apoptosis, but does not affect CD95-induced apoptosis of the Jurkat T cell line and hepatocytes (39–41). In our studies hypoxia inhibited Dex-induced, but not CD95/Fas-induced, apoptosis, suggesting that hypoxia may be acting by affecting the Apaf-1/capase 9 complex formation. The ATP requirement for Apaf-1/caspase 9 complex formation raises the possibility that a reduction of ATP levels during hypoxia may be responsible for the inhibition of Dex-induced thymocyte apoptosis.

The kinetics of hypoxia-induced ATP reduction in resting thymocytes are, however, not known. Chemical anoxia of rat thymocytes induced by oligomycin or antimycin A treatment reduces ATP levels by about 70–80% within 2 h (39). Similar results have been shown for rat fibroblasts treated with antimycin A (42). In hypoxia-exposed human embryonic kidney cells, on the other hand, ATP levels are not reduced before 12 h (43). Our studies show that hypoxia inhibits $\Delta\Psi_m$ by 2 h and apoptosis by 5 h. Rotenone reduces ATP levels in rat thymocytes rapidly within 2 h, and our data that show that rotenone inhibits Dex-induced apoptosis could be interpreted by the effect rotenone has on ATP levels. Taken together, the above could make a case for hypoxia's inhibitory effect on Dex-induced apoptosis being attributed to ATP depletion under hypoxia. Our observations, however, showing that TMPO, an electron spin trap, inhibits Dex-induced apoptosis argue against this. We cannot, however, at present exclude that hypoxia's inhibitory effect on thymocyte apoptosis is mediated through a drop in the level of ATP that limits formation of the Apaf-1/caspase 9 complex.

Finally, hypoxia may be affecting Dex-induced apoptosis by inducing intracellular acidification. The role of intracellular pH in apoptosis is unclear at present, with data suggesting that both acidification and alkalinization promote apoptosis (44, 45). Caspase activation by cytochrome *c* is maximal in acidic cytoplasmic pH (45). Since Dex-induced apoptosis is dependent on Apaf-1/cytochrome *c* (36–38), and hypoxia induces a rapid drop in pH, hypoxia should enhance Dex-induced apoptosis and not inhibit it as we observed. Alkalinization, on the other hand, has been reported to enhance apoptosis by inducing conformational changes and the translocation of Bax to the mitochondria (44). Acidification can inhibit this translocation of Bax and apoptosis (44). Since thymocytes undergo cytosolic alkalinization during glucocorticoid-induced apoptosis (46, 47), hypoxia may be inhibiting Dex-induced apoptosis by counteracting this alkalinization. Although this could be a plausible explanation for how hypoxia inhibits Dex-induced apoptosis, it is irreconcilable with the fact that Bax is not required for glucocorticoid-induced thymocyte apoptosis (48). Further studies examining cytosolic pH changes induced by hypoxia and their role in Dex-induced apoptosis are required to address these questions.

O_2-dependent apoptosis could play an important role in human disease. Ischemia reperfusion injury has been proposed as a major mechanism of cellular injury in myocardial postischemic injury (6, 49, 50). In addition, O_2-dependent apoptosis may be involved in tumor development (51) and neurological degenerative disorders such as amyotropic lateral sclerosis (52, 53). Elucidating the O_2-dependent signaling of apoptosis may prove useful in understanding the pathophysiology of these conditions and designing novel therapeutics. Finally, O_2-dependent apoptosis may be important during thymocyte development, since glucocorticoids may be involved in the elimination of unselected thymocytes in the thymus (54). Recent studies have shown that glucocorticoids set the threshold for thymocyte selection and that diminished glucocorticoid signaling in the thymus results in holes in the peripheral T cells repertoire (55, 56). This raises the possibility that hypoxia, which inhibits at least part of the outcome of Dex signaling, i.e., apoptosis, may also interfere with the antagonizing effect glucocorticoids have on TCR-mediated signaling and thus also affect thymic selection. These observation raise intriguing questions of how hypoxia and O_2^- generation affect the immune system.

In conclusion, we have presented evidence for the existence of two independent apoptosis pathways that are distinguished by hypoxic culture (oxygen dependent and oxygen independent). The events affected by hypoxia in Dex-treated thymocytes are upstream of mitochondrial PT, the earliest evidence of thymocyte irreversible death. Both Dex and anti-CD95 apoptosis were dependent on the activation of ICE-like proteases, suggesting that these independent pathways converge at this point. Hypoxia also inhibited caspase-3-like protease activity in Dex-treated thymocytes, suggesting that the events regulated by hypoxia are upstream of the caspase-3-like proteases. Further studies should concentrate on identifying the biochemical events sensitive to hypoxia during O_2-dependent signaling of apoptosis.

References

1. Cohen, J. J. 1993. Apoptosis. *Immunol. Today 14:126.*
2. Kroemer, G., N. Zamzami, and S. A. Susin. 1997. Mitochondrial control of apoptosis. *Immunol. Today 18:44.*
3. Buttke, T. M., and P. A. Sandstrom. 1994. Oxidative stress as a mediator of apoptosis. *Immunol. Today 15:7.*
4. Matthews, N., M. L. Neale, S. K. Jackson, and J. M. Stark. 1987. Tumour cell killing by tumour necrosis factor: inhibition by anaerobic conditions, free-radical scavengers and inhibitors of arachidonate metabolism. *Immunology. 62:153.*
5. Larrick, J. W., and S. C. Wright. 1990. Cytotoxic mechanism of tumor necrosis factor-α. *FASEB J. 4:3215.*
6. Halliwell, B., and J. M. C. Gutteridge. 1990. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol. 186:1.*
7. Sandstrom, P. A., M. D. Mannie, and T. M. Buttke. 1994. Inhibition of activation-induced death in T cell hybridomas by thiol antioxidants: oxidative stress as a mediator of apoptosis. *J. Leukocyte Biol. 55:221.*
8. Torres-Roca, J. F., C. Amatore, and M. L. Gougeon. 1995. The early production of a reactive oxygen intermediate mediates apoptosis in dexamethasone-treated thymocytes. *Cell Death Differ. 2:309.*
9. Brüne, B., P. Hartzell, P. Nicotera, and S. Orrentus. 1991. Spermine prevents endonuclease activation and apoptosis in thymocytes. *Exp. Cell Res. 195:323.*
10. Slater, A. F., C. S. Nobel, E. Maellaro, J. Bustamante, M. Kimland, and S. Orrenius. 1995. Nitrone spin traps and a nitroxide antioxidant inhibit a common pathway of thymocyte apoptosis. *Biochem J. 306:771.*
11. Lennon, S. V., S. J. Martin, and T. G. Cotter. 1991. Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell Prolif. 24:203.*
12. Jacobson, M. D., and M. C. Raff. 1995. Programmed cell death and Bcl-2 protection in very low oxygen. *Nature 374:814.*
13. Muschel, R. J., E. J. Bernhard, L. Garza, W. G. McKenna, and C. J. Koch. 1995. Induction of apoptosis at different oxygen tensions: evidence that oxygen radicals do not mediate apoptotic signaling. *Cancer Res. 55:995.*
14. McLaughlin, K. A., B. A. Osborne, and R. A. Goldsby. 1996. The role of oxygen in thymocyte apoptosis. *Eur. J. Immunol. 26:1170.*
15. Costantini, P., B. V. Chernyak, V. Petronilli, and P. Bernardi. 1996. Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. *J. Biol. Chem. 271:6746.*
16. Marchetti, P., D. Decaudin, A. Macho, N. Zamzami, T. Hirsch, S. A. Susin, and G. Kroemer. 1997. Redox regulation of apoptosis: impact of thiol oxidation status on mitochondrial function. *Eur. J. Immunol. 27:289.*
17. Yang, J., X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. I. Peng, D. P. Jones, and X. Wang. 1997. Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science 275:1129.*
18. Kluck, R. M., E. Bossy-Wetzel, D. R. Green, and D. D. Newmeyer. 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science 275:1132.*
19. Katsikis, P. D., E. S. Wunderlich, C. A. Smith, L. A. Herzenberg, and L. A. Herzenberg. 1995. Fas antigen stimulation induces marked apoptosis of T lymphocytes in human immunodeficiency virus-infected individuals. *J. Exp. Med. 181:2029.*
20. Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, J. L. Vayssiere, P. X. Petit, and G. Kroemer. 1995. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J. Exp. Med. 181:1661.*
21. Petit, P. X., H. Lecoeur, E. Zorn, C. Dauguet, B. Mignotte, and M. L. Gougeon. 1995. Alterations in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. *J. Cell Biol. 130:157.*
22. Moore, W. A., and R. A. Kautz. 1986. Data analysis in flow cytometry. In *Handbook of Experimental Immunology.* D. M. Weir, L. A. Herzenberg, C. Blackwell, and L. A. Herzenberg, eds. Blackwell, Oxford, p. 30.
23. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
24. Nicholson, D. W., A. Ali, and N. A. Thornberry. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature 376:37.*
25. D'Amadio, F., O. Zollo, R. Moraca, E. Ayroldi, S. Bruscoli, A. Bartoli, L. Cannarile, G. Migliorati, and C. Riccardi. 1997. A new dexamethasone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death. *Immunity 7:803.*
26. Marchetti, P., M. Castedo, S. A. Susin, N. Zamzami, T. Hirsch, A. Macho, A. Haeffner, F. Hirsch, M. Geuskens, and G. Kroemer. 1996. Mitochondrial permeability transition is a central coordinating event of apoptosis. *J. Exp. Med. 184:1155.*

27. Zamzami, N., P. Marchetti, M. Castedo, D. Decaudin, A. Macho, T. Hirsch, S. A. Susin, P. X. Petit, B. Mignotte, and G. Kroemer. 1995. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J. Exp. Med. 182:367.*
28. Hochachka, P. W., L. T. Buck, C. J. Doll, and S. C. Land. 1996. Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. USA 93:9493.*
29. Liu, X., C. N. Kim, J. Yang, R. Jemmerson, and X. Wang. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell 86:147.*
30. Susin, S. A., N. Zamzami, M. Castedo, T. Hirsch, P. Marchetti, A. Macho, E. Daugas, M. Geuskens, and G. Kroemer. 1996. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J. Exp. Med. 184:1331.*
31. Alam, A., M. Y. Braun, F. Hartgers, S. Lesage, L. Cohen, P. Hugo, F. Denis, and R. P. Sekaly. 1997. Specific activation of the cysteine protease CPP32 during the negative selection of T cells in the thymus. *J. Exp. Med. 186:1503.*
32. Furukawa, K., S. Estus, W. Fu, R. J. Mark, and M. P. Mattson. 1997. Neuroprotective action of cycloheximide involves induction of bcl-2 and antioxidant pathways. *J. Cell Biol. 136:1137.*
33. Sentman, C. L., J. R. Shutter, D. Hockenbery, O. Kanagawa, and S. J. Korsmeyer. 1991. Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell 67:879.*
34. Strasser, A., A. W. Harris, D. C. Huang, P. H. Krammer, and S. Cory. 1995. Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *EMBO J. 14:6136.*
35. Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri, and X. Wang. 1997. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell 91:479.*
36. Kuida, K., T. F. Haydar, C. Y. Kuan, Y. Gu, C. Taya, H. Karasuyama, M. S. Su, P. Rakic, and R. A. Flavell. 1998. Reduced apoptosis and cytochrome *c*-mediated caspase activation in mice lacking caspase 9. *Cell 94:325.*
37. Hakem, R., A. Hakem, G. S. Duncan, J. T. Henderson, M. Woo, M. S. Soengas, A. Elia, J. L. de la Pompa, D. Kagi, W. Khoo, et al. 1998. Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell 94:339.*
38. Yoshida, H., Y. Y. Kong, R. Yoshida, A. J. Elia, A. Hakem, R. Hakem, J. M. Penninger, and T. W. Mak. 1998. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell 94:739.*
39. Stefanelli, C., F. Bonavita, I. Stanic, G. Farruggia, E. Falcieri, I. Robuffo, C. Pignatti, C. Muscari, C. Rossoni, C. Guarnieri, et al. 1997. ATP depletion inhibits glucocorticoid-induced thymocyte apoptosis. *Biochem. J. 322:909.*
40. Ferrari, D., A. Stepczynska, M. Los, S. Wesselborg, and K. Schulze-Osthoff. 1998. Differential regulation and ATP requirement for caspase-8 and caspase-3 activation during CD95- and anticancer drug-induced apoptosis. *J. Exp. Med. 188:979.*
41. Latta, M., G. Kunstle, M. Leist, and A. Wendel. 2000. Metabolic depletion of ATP by fructose inversely controls CD95- and tumor necrosis factor receptor 1-mediated hepatic apoptosis. *J. Exp. Med. 191:1975.*
42. Formigli, L., L. Papucci, A. Tani, N. Schiavone, A. Tempestini, G. E. Orlandini, S. Capaccioli, and S. Z. Orlandini. 2000. Aponecrosis: morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. *J. Cell Physiol. 182:41.*
43. Ozawa, K., K. Kuwabara, M. Tamatani, K. Takatsuji, Y. Tsukamoto, S. Kaneda, H. Yanagi, D. M. Stern, Y. Eguchi, Y. Tsujimoto, et al. 1999. 150-kDa oxygen-regulated protein (ORP150) suppresses hypoxia-induced apoptotic cell death. *J. Biol. Chem. 274:6397.*
44. Khaled, A. R., K. Kim, R. Hofmeister, K. Muegge, and S. K. Durum. 1999. Withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria through a rise in intracellular pH. *Proc. Natl. Acad. Sci. USA 96:14476.*
45. Matsuyama, S., J. Llopis, Q. L. Deveraux, R. Y. Tsien, and J. C. Reed. 2000. Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nat. Cell. Biol. 2:318.*
46. Tsao, N., and H. Y. Lei. 1996. Activation of the Na^+/H^+ antiporter, $Na^+/HCO_3^-/CO_3^{2-}$ cotransporter, or Cl^-/HCO_3^- exchanger in spontaneous thymocyte apoptosis. *J. Immunol. 157:1107.*
47. Lei, H. Y., M. J. Tong, and N. Tsao. 1997. Intracellular alkalinization in dexamethasone-induced thymocyte apoptosis. *Apoptosis 2:304.*
48. Knudson, C. M., K. S. Tung, W. G. Tourtellotte, G. A. Brown, and S. J. Korsmeyer. 1995. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science 270:96.*
49. Gottlieb, R. A., K. O. Burleson, R. A. Kloner, B. M. Babior, and R. L. Engler. 1994. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J. Clin. Invest. 94:1621.*
50. Thompson, C. B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science 267:1456.*
51. Graeber, T. G., C. Osmanian, T. Jacks, D. E. Housman, C. J. Koch, S. W. Lowe, and A. J. Giaccia. 1996. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature 379:88.*
52. Troy, C. M., L. Stefanis, L. A. Greene, and M. L. Shelanski. 1997. Mechanisms of neuronal degeneration: a final common pathway? *Adv. Neurol. 72:103.*
53. Troy, C. M., D. Derossi, A. Prochiantz, L. A. Greene, and M. L. Shelanski. 1996. Downregulation of Cu/Zn superoxide dismutase leads to cell death via the nitric oxide-peroxynitrite pathway. *J. Neurosci. 16:253.*
54. Weissman, I. L. 1994. Developmental switches in the immune system. *Cell 76:207.*
55. Vacchio, M. S., J. Y. Lee, and J. D. Ashwell 1999. Thymus-derived glucocorticoids set the thresholds for thymocyte selection by inhibiting TCR-mediated thymocyte activation. *J. Immunol. 163:1327.*
56. Lu, F. W. M., K. Yasutomo, G. B. Goodman, L. McHeyzer-Williams, M. G. McHeyzer-Williams, R. N. Germain, and J. D. Ashwell 2000. Thymocyte resistance to glucocorticoids leads to antigen-specific unresponsiveness due to holes in the T cell repertoire. *Immunity 12:183.*