

# Critical Role for Mitochondrial Oxidative Phosphorylation in the Activation of Tumor Suppressors Bax and Bak

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**Background:** Activation of Bax and Bak, which act to permeabilize the mitochondrial membrane, is an essential step in the cell death response and therefore in the suppression of tumorigenesis. However, the mechanisms that regulate activation are poorly understood. **Methods:** Bax and Bak activation (conformational change and dimerization) was monitored in Rat-1 fibroblasts and human cancer cells subjected to endoplasmic reticulum (ER) stress, DNA damage, or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) treatment. Pharmacologic inhibitors of reactive oxygen species production, electron transport in the respiratory chain, oxidative phosphorylation, and appropriate controls were used to identify potential modes by which Bax and Bak activation and the cell death response are controlled. The oligomerization state of Bax and Bak was determined by cross-linking and subsequent immunoblot analysis; Bax conformational change was analyzed by immunoprecipitation and immunoblotting with an antibody specific for the active conformation. Cell death was evaluated by dye exclusion. **Results:** In both fibroblasts and human cancer cells subjected to cell death stimuli, inhibition of oxidative phosphorylation by use of antimycin A or oligomycin prevented ER stress-, DNA damage-, and TNF- $\alpha$ -induced Bax and Bak activation and cell death (UV-induced Rat-1 cell death at 15 hours: control, mean = 33.6%, 95% confidence interval [CI] = 18.8% to 48.4%; antimycin A, mean = 10.0%, 95% CI = 0% to 21.7%; oligomycin, mean = 13.1%, 95% CI = 5.7% to 20.5%; tunicamycin-induced MCF-7 cell death at 9 hours: control, mean = 29.2%, 95% CI = 21.6% to 36.8%; antimycin A, mean = 15.3%, 95% CI = 0.8% to 29.8%; oligomycin, mean = 11.5%, 95% CI = 3.9% to 19.1%; TNF- $\alpha$ -induced MCF-7 cell death at 6 hours: control, mean = 24.0%, 95% CI = 12.6% to 35.4%; antimycin A, mean = 8.9%, 95% CI = 3.9% to 13.9%; oligomycin, mean = 13.3%, 95% CI = 10.4% to 16.2%). Increasing and decreasing glycolytic adenosine triphosphate production, by adding glucose and 2-deoxy-D-glucose to the cell growth medium, respectively, neither reversed nor recapitulated, respectively, the effect of compromised oxidative phosphorylation on Bax and Bak activation. **Conclusion:** Oxidative phosphorylation is required for the activation of Bax and Bak and cell death triggered by disparate death stimuli. The reliance of tumor cells on glycolysis in preference to oxidative phosphorylation even under normoxic conditions (Warburg effect) may therefore be a potential means by which these cells evade programmed cell death. [J Natl Cancer Inst 2006;98:1462-73]

The Bcl-2 family of proteins plays a critical role in the regulation of programmed cell death and tumorigenesis. Proteins in

this family control cell death by altering the permeability of the outer mitochondrial membrane. This membrane sequesters death-signaling molecules (e.g., cytochrome c and second mitochondrially-derived activator of caspases [Smac]/Direct IAP [inhibitor of apoptosis] binding protein with low pI [DIABLO]) from cognate effectors present in the cytosol (1,2). Genetic knockout studies have revealed that the Bax-like Bcl-2 family members Bax and Bak are essential for cell death triggered by diverse stimuli that act on mitochondria (3), and current models (1,2) assume that Bax and Bak play a central role in mitochondrial membrane permeabilization. This function gives them a critical role in the inhibition of cellular transformation and tumorigenesis, a role that has been demonstrated in vitro (4) and in animal models (5,6) and one whose importance is suggested by the finding that Bax and Bak undergo genetic and epigenetic inactivations in some human tumors (7). However, it is unclear whether the intact Bax and Bak proteins expressed in other human tumors are functionally inactivated and, if so, how.

In response to death stimuli, and before membrane permeabilization, Bax and Bak undergo a set of activation steps (1,2)—mitochondrial translocation, stable insertion (integration) into the mitochondrial membrane, conformational change, and oligomerization (the first two steps are not required in the case of Bak, which exists as an integral membrane protein of the mitochondrion even in healthy cells). In contrast to the considerable efforts to understand how activated Bax and Bak function to permeabilize the mitochondrial membrane, there has been little investigation of the mechanisms that regulate activation of Bax and Bak. Accumulating evidence suggests that the Bcl-2 homology 3 (BH3)-only proteins, proapoptotic members of the Bcl-2 family that contain only the BH3 domain out of the four BH domains, play a critical role in Bax and Bak activation by a variety of death stimuli (1,2). However, it is unclear how most of these proteins regulate Bax and Bak in the absence of apparent physical interaction (1,2). Additional mechanisms for regulation of Bax and Bak activation remain completely uncharacterized.

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See "Notes" following "References."

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In this report, we have monitored the activation of Bax and Bak in several cell death models including those induced by endoplasmic reticulum (ER) stress and DNA damage, known to trigger mitochondria-mediated cell death in Bax- and Bak-dependent manner (3). We used pharmacologic inhibitors and appropriate controls to identify modes by which the activation of these proteins and hence the cell death response and cell transformation are controlled.

## MATERIALS AND METHODS

### Pharmacologic Inducers and Inhibitors of Cell Death, Antibodies, and Reagents

Etoposide (diluted in dimethylsulfoxide [DMSO], 30 mg/mL), rotenone (diluted in DMSO, 0.5 mg/mL), antimycin A (diluted in ethanol, 50 mg/mL), indomethacin (diluted in ethanol, 20 mg/mL), allopurinol (diluted in 1 M NaOH, 50 mg/mL), D-neopterin (diluted in distilled water, 0.5 mg/mL), phenylarsine oxide (diluted in DMSO, 50 mg/mL), aurovertin B (diluted in chloroform, 54 mM), Hoechst 33342 (diluted in DMSO, 100 mM), standard Dulbecco's modified Eagle medium (DMEM), glucose-free DMEM base, sodium pyruvate, L-glutamine, and sodium bicarbonate were purchased from Sigma (St Louis, MO). Fetal bovine serum (FBS) was from Immuno-Biological Laboratories (Takasaki, Japan). Glucose and 2-deoxy-D-glucose (2-deoxy-D-glucose, diluted in distilled water, 2 M) were from Wako (Tokyo, Japan). Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (diluted in phosphate-buffered saline [PBS] containing 0.1% bovine serum albumin, 0.2  $\mu$ M) was from Genzyme-Techne (Minneapolis, MI). Tunicamycin (diluted in DMSO, 20 mM), brefeldin A (diluted in ethanol, 1 mg/mL), Boc-Asp-fmk (BAF, diluted in DMSO, 50 mM), cycloheximide (diluted in ethanol, 10 mg/mL), N-acetylcysteine (diluted in distilled water, 1 M), Trolox (diluted in ethanol, 500 mg/mL), and monoclonal anti-Bax antibody (6A7) were from Calbiochem (San Diego, CA). Anti-Bip (GRP78) antibody was from Stressgen (Ann Arbor, MI). Anti-C/EBP-homologous protein (CHOP) (GADD157) antibody, polyclonal anti-Bak antibody (G-23), polyclonal anti-Bax antibody (N-20), anti-Bcl-2 antibody (N-19), anti-p53 antibody, anti-actin antibody, and horseradish peroxidase-conjugated anti-mouse, -rabbit, and -goat IgG secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-Bak antibody (NT) was from Upstate Biotechnology (Charlottesville, VA). Anti-cytochrome c antibody was from BD Pharmingen (San Jose, CA). Bismaleimido-hexane (BMH, diluted in DMSO, 15 mM) and bicinchoninic acid (BCA) protein assay kit were from Pierce (Rockford, IL). ECL western blotting detection reagents and Protein G-Sepharose 4 Fast Flow were from Amersham Pharmacia Biotech (Piscataway, NJ). Adenosine triphosphate (ATP) bioluminescence kit was from Roche (Indianapolis, IL). 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA, diluted in DMSO, 20 mM) was from Molecular Probes (Eugene, OR). Effectene was from Qiagen (Hilden, Germany).

### Cell Lines and Cell Culture

Rat-1 rat fibroblasts (kind gift from Dr Kaoru Segawa, Keio University, Tokyo, Japan), MCF-7 human breast cancer cells (kind gift from Dr Shigeaki Kato, Institute of Molecular and Cellular Biosciences, the University of Tokyo, Tokyo, Japan), and

HepG2 human hepatocellular carcinoma cells (kind gift from Dr Akinori Sugiyama, Kyushu University of Health and Welfare, Miyazaki, Japan) were grown, unless otherwise indicated, in the standard DMEM (glucose = 25 mM) supplemented with 10% FBS and antibiotics (100 U/mL penicillin, 100  $\mu$ g/mL streptomycin). DMEM with increasing concentrations of glucose (25, 50, and 75 mM) was prepared by adding glucose to glucose-free DMEM base. Solutions were autoclaved and supplemented with 1 mM sodium pyruvate, 4 mM L-glutamine, 0.37% sodium bicarbonate, 10% FBS, and antibiotics (100 U/mL penicillin, 100  $\mu$ g/mL streptomycin).

### Establishment of Stable Transfectants

Stable Rat-1 transfectants were established by transfecting Rat-1 cells with pcDNA3 vectors using Effectene and selecting transfectants with G418. The vectors were modified to express wild-type Bcl-2, Bcl-acta (Bcl-2 with its C terminal insertion sequence replaced by an equivalent sequence from ActA) (8), Bcl-cb5 (Bcl-2 with its C terminus insertion sequence replaced by an equivalent sequence of cytochrome b5) (8), c-Myc, or activated RasV12. For each transfectant, multiple clones expressing the intended protein were subjected to subsequent analyses, and essentially similar results were obtained from the multiple clones. For each transfectant, therefore, results from one of the clones are presented.

### Apoptosis, Cell Death, and Survival Assays

Unless otherwise indicated, apoptosis and cell death were induced by one of the following death stimuli: 70  $\mu$ M tunicamycin, 20 J/m<sup>2</sup> UV-C (UV-C was delivered automatically in Spectrolinker XL-1000 [Spectronics Corporation, Westburg, NY], after aspiration of culture medium), 20  $\mu$ M etoposide, 2  $\mu$ g/mL brefeldin A, and 15 ng/mL TNF- $\alpha$  plus 5  $\mu$ g/mL cycloheximide. The pan-caspase inhibitor BAF was added to the culture medium 2 hours before treatment with cell death stimuli. Inhibitors of the mitochondrial respiratory chain and oxidative phosphorylation (i.e., antimycin A, rotenone, oligomycin, and aurovertin B) were added to the culture medium just before treatment with cell death stimuli, except when the stimulus was UV irradiation. When cell death was induced with UV, these inhibitors were added at the time cells were replenished with fresh medium just after UV irradiation. Other inhibitors (i.e., indomethacin, allopurinol, D-neopterin, phenylarsine oxide, BAF, Trolox, and NAC) were added to the culture medium 1 hour before treatment with cell death stimuli. Apoptosis and cell death were assayed at the time points indicated in the figure legends after treatment with cell death stimuli. Apoptosis was assessed by nuclear staining with 100  $\mu$ M Hoechst 33342. In brief, cells stained with the Hoechst dye were examined under a fluorescence microscope, and the percentage of apoptotic cells, as defined by condensed and fragmented nuclei, was determined (a total of 1000 cells per dish were assayed). Cell death was detected based on cells' inability to exclude vital dyes. In brief, both detached and adherent cells were collected by trypsin treatment and suspended in PBS. Then 20  $\mu$ L of the cell suspension was mixed with 20  $\mu$ L of 0.5% trypan blue dye solution, and the percentage of dead cells unable to exclude the dye was determined under a phase-contrast microscope ( $\times$ 100 magnification, 10 visual fields per dish). For the survival assay, 48 hours after treatment with cell death stimuli, dead cells were washed away with three rinses of PBS, and the

surviving cells attached to the culture dish were counted (rounded and/or fragmented cells were excluded) under  $\times 200$  magnification in randomly selected 25 visual fields per dish.

### Measurement of Levels of Intracellular Reactive Oxygen Species

Rat-1 cells were cultured in the presence of 20  $\mu\text{M}$  2',7'-dichlorodihydrofluorescein diacetate (DCFDA) for 30 minutes. After the culture medium was removed, cells were washed twice with PBS, replenished with 3 mL of Hanks' balanced salt solution per 60-mm dish, and examined under a fluorescence microscope. The fluorescence images were captured by the SPOT system (Diagnostic instruments, Sterling Heights, MI) using a manual protocol (single exposure, exposure time 0.2 second). Image analysis and quantitation of mean DCFDA fluorescence intensity of each cell was done by using IP Lab software (Scanalytics, Rockville, MD). At least 1000 cells were examined for each data point.

### Cell Fractionation and Immunoblotting

To prepare whole-cell lysates, both detached and adherent cells were collected from the dish by gentle scraping and washed twice with PBS following centrifugation at 2000g. Pelleted cells were lysed by the lysis buffer (0.5 M Tris-HCl [pH 6.8], 10% glycerol, 2% sodium dodecyl sulfate [SDS]) and sonicated for 1 minute. For cell fractionation, pelleted cells were permeabilized for 1 minute in isotonic buffer A (10 mM HEPES [pH 7.5], 10 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 250 mM sucrose) containing 0.05% digitonin for 1 minute at room temperature and then centrifuged at 15 000 g for 10 minutes. The supernatant (cytosolic fraction) and the pellet (mitochondrial fraction) were collected, and the pellet was further lysed in lysis buffer followed by sonication. For immunoblotting, protein concentration of the lysates was determined using the BCA protein assay kit according to the manufacturer's instructions. Equal amounts of protein were separated by 10%–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to a nitrocellulose membrane using a semidry transfer system. Membranes were blocked at room temperature for 1 hour in the blocking buffer (5% skim milk in PBS) and then incubated with a primary antibody diluted in antibody buffer (5% skim milk and 0.1% Tween 20 in PBS, 1 : 500 dilution) at room temperature for 2 hours. Then membranes were washed three times with the antibody buffer, incubated with a peroxidase-conjugated secondary antibody diluted in the antibody buffer (1 : 1000 dilution) at room temperature for 1 hour, and washed three times with PBS containing 0.1% Tween 20. Membrane-bound antibodies were visualized by ECL western blotting detection reagents.

### In Vitro Cross-linking for Detection of Bax and Bak Oligomers

In vitro cross-linking of associated Bax or Bak monomers was performed according to the method of Sundararajan et al. (9) with some modifications. In brief, cells were permeabilized in cross-linking buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM HEPES [pH 7.4], 0.05% digitonin), and then BMH was added to a final concentration of 7 mM. After incubation for 30 minutes at room temperature, the lysate was centrifuged at 15 000g for 10 minutes. The pellet was lysed in lysis buffer

(62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS), sonicated, and centrifuged at 15 000g for 15 minutes. After determination of the protein concentration using the BCA protein assay kit, the lysate was subjected to immunoblot analysis using polyclonal anti-Bax (N-20) and anti-Bak (G-23 or NT) antibodies.

### Immunoprecipitation

The active conformer of Bax was purified by immunoprecipitation and then detected by immunoblotting. In brief, Rat-1 cells were lysed in CHAPS buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 1% CHAPS), and lysates containing 1 mg protein (as determined by the BCA protein assay kit) were brought to a final volume of 400  $\mu\text{L}$  with CHAPS buffer. An 8- $\mu\text{L}$  sample of this solution (termed Pre-IP lysate) was removed. Five micrograms of monoclonal anti-Bax antibody (6A7) was added to the remaining solution, and samples were rotated at 4 °C overnight. After addition of 40  $\mu\text{L}$  Protein G–Sepharose 4 Fast Flow, samples were rotated for another 2 hours. The samples were then centrifuged at 3000g for 1 minute, and immunoprecipitates were eluted in 4 $\times$  SDS sample buffer (250 mM Tris-HCl [pH 6.8], 40% glycerol, 8% SDS, 0.05% bromphenol blue, and 20% 2-mercaptoethanol). The immunoprecipitates and the Pre-IP lysates were separated by SDS–PAGE and subjected to immunoblotting with polyclonal anti-Bax antibody (N-20), followed by incubation with a peroxidase-conjugated secondary antibody. Blots were visualized using ECL western blotting detection reagents.

### Measurement of Cellular ATP

Cellular ATP concentrations were determined based on the luciferin–luciferase reaction using an ATP bioluminescence kit. After detached (i.e., dead) cells were washed away from the dish by rinsing with PBS, adherent (i.e., live) cells were harvested by trypsinization and suspended in 1 mL of PBS. The cell density of the cell suspension was determined by counting cell numbers under a phase-contrast microscope ( $\times 100$  magnification, 10 visual fields). A 50- $\mu\text{L}$  sample of the cell suspension was removed and mixed with cell lysis buffer (50  $\mu\text{L}$ ) from the kit and subsequently with luciferase reagent from the kit (100  $\mu\text{L}$ ). Luminescence was then quantified with a Mini Lumat LB 9506 luminometer (Berthold Technologies, Bad Wildbad, Germany). The average cell density (determined from at least three aliquots of a single-cell suspension) and luminescence were used to calculate ATP content per cell.

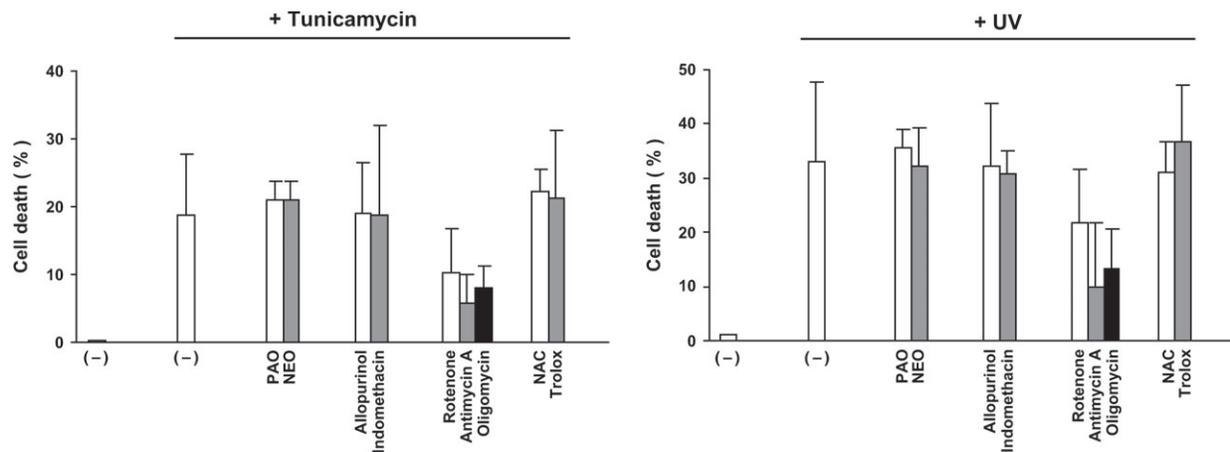
### Statistical Analysis

For all experiments with quantitative results, the data are expressed as the mean and 95% confidence interval (CI) from three identical experiments carried out independently. Means and 95% confidence intervals were calculated using Microsoft Office Excel 2003 software (Microsoft Corp., Redmond, WA).

## RESULTS

### Rat-1 Fibroblasts Exposed to ER Stress and DNA Damage Are a Model for Bax Activation and Cell Death

ER stress and DNA damage induce Bax- and Bak-dependent cell death in rodent fibroblasts (3). In preliminary experiments,



**Fig. 1.** Essential role for mitochondrial oxidative phosphorylation, but not reactive oxygen species, in Rat-1 cell death induced by endoplasmic reticulum stress and DNA damage. Rat-1 cells treated without or with 70  $\mu\text{M}$  tunicamycin (**left panel**) or 20  $\text{J}/\text{m}^2$  UV (**right panel**) in the absence or presence of the indicated inhibitors were assayed for cell death 21 and 15 hours after tunicamycin and UV treatments, respectively. The data represent the mean values of three independent experiments,

and **error bars** correspond to the upper 95% confidence intervals. The inhibitors were used at the following concentrations: phenylarsine oxide (PAO), 0.3  $\mu\text{M}$ ; D-neopterin (NEO), 0.5  $\mu\text{M}$ ; allopurinol, 3  $\mu\text{M}$ ; indomethacin, 3  $\mu\text{M}$ ; rotenone, 0.1  $\mu\text{M}$ ; antimycin A, 7  $\mu\text{g}/\text{mL}$ ; oligomycin, 3  $\mu\text{g}/\text{mL}$ ; *N*-acetylcysteine (NAC), 3 mM; Trolox, 0.6  $\mu\text{M}$ .

we observed that ER stress inducers, such as tunicamycin and brefeldin A, and DNA-damaging agents, such as UV and etoposide, induced cell death in Rat-1 fibroblasts that was inhibited by overexpression of Bcl-2 (Supplementary Fig. 1, A; available at: <http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue20>). This was additional evidence that the cell death pathway in these models involved Bax activation. To further characterize the models, we treated Rat-1 cells exposed to tunicamycin and UV with the pan-caspase inhibitor BAF. The extent of cell death was largely insensitive to the caspase inhibitor, whereas apoptosis was almost completely prevented by BAF treatment (Supplementary Fig. 1, B; available at: <http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue20>). This suggested that cell death was occurring independently of apoptosis in these models.

### Essential Role for Oxidative Phosphorylation but Not Reactive Oxygen Species in Cell Death Induced by ER Stress and DNA Damage

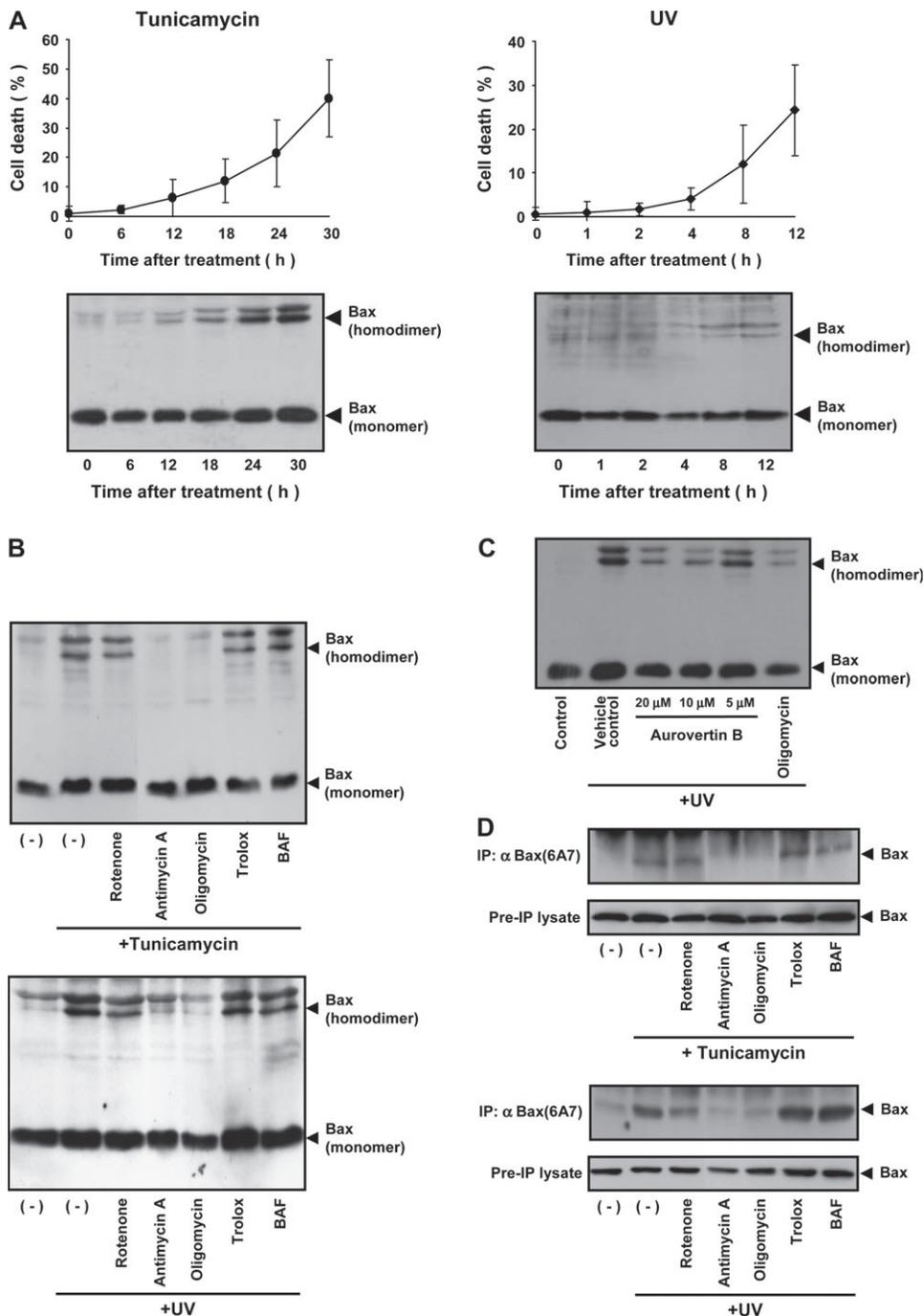
Because reactive oxygen species have been implicated in the regulation of caspase-independent cell death (10), we examined their role in ER stress- and DNA damage-induced cell death by treating Rat-1 cells with a series of pharmacologic inhibitors to modulate intracellular levels of reactive oxygen species. Treatment of Rat-1 cells with 70  $\mu\text{M}$  tunicamycin caused an increase in reactive oxygen species production as evidenced by the increased fluorescence of the reactive oxygen species-sensitive dye DCFDA, and 19.1% (95% CI = 10.1% to 28.1%) of the cells underwent cell death within 21 hours (Fig. 1 and data not shown). Pretreatment of the cells with inhibitors of reactive oxygen species-generating enzymes, either reduced nicotinamide adenine dinucleotide phosphate oxidase inhibitors (phenylarsine oxide or D-neopterin) or xanthine oxidase inhibitors (allopurinol or indomethacin), before tunicamycin exposure did not reduce either tunicamycin-induced reactive oxygen species production (data not shown) or cell death (Fig. 1). However, tunicamycin-induced Rat-1 cell death at 21 hours was reduced to 10.3% (95% CI = 3.9% to 16.7%) by treatment of cells with the mitochondrial

respiratory chain inhibitor rotenone (0.1  $\mu\text{M}$ ) and to 6.1% (95% CI = 1.8% to 10.4%) by treatment with another inhibitor of electron transport in the respiratory chain, antimycin A (7  $\mu\text{g}/\text{mL}$ ). The effect of these electron transport inhibitors was consistent with the idea that reactive oxygen species generated in the respiratory chain might play an important role in tunicamycin-induced Rat-1 cell death. However, the antioxidants *N*-acetylcysteine and Trolox failed to inhibit cell death induced by tunicamycin (Fig. 1), even though Trolox blocked the accumulation of reactive oxygen species no less efficiently than rotenone and antimycin A (data not shown). Treatment of cells with oligomycin (3  $\mu\text{g}/\text{mL}$ ), a specific inhibitor of the  $F_0$ -subunit of  $F_0F_1$ -adenosine triphosphatase (ATPase) and thus an inhibitor of oxidative phosphorylation, efficiently reduced tunicamycin-induced Rat-1 cell death at 21 hours to 8.4% (95% CI = 5.2% to 11.6%) (Fig. 1).

In a parallel series of experiments, we tested the effect of these inhibitors when cell death of Rat-1 fibroblasts was induced by exposure to UV. When Rat-1 cells were treated with 20  $\text{J}/\text{m}^2$  UV, 33.6% (95% CI = 18.8% to 48.4%) of the cells underwent death within 15 hours after UV treatment. This UV-induced Rat-1 cell death was reduced to 22.6% (95% CI = 12.7% to 32.6%) by 0.1  $\mu\text{M}$  rotenone, to 10.0% (95% CI = 0% to 21.7%) by 7  $\mu\text{g}/\text{mL}$  antimycin A, and to 13.1% (95% CI = 5.7% to 20.5%) by 3  $\mu\text{g}/\text{mL}$  oligomycin. Phenylarsine oxide, D-neopterin, allopurinol, indomethacin, *N*-acetylcysteine, and Trolox did not reduce UV-induced Rat-1 cell death (Fig. 1). Thus, in relative terms, the effect of all the inhibitors was similar whether cell death was induced by ER stress or DNA damage. In cell survival assays, antimycin A and oligomycin, but not Trolox, increased survival of Rat-1 cells treated with tunicamycin or UV (Supplementary Fig. 2, available at: <http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue20>).

To confirm that tunicamycin and UV treatment of Rat-1 fibroblasts acts specifically to induce ER stress and DNA damage, respectively, we examined levels of some proteins whose expression increases selectively in response to these stresses. Tunicamycin treatment caused increased expression of Bip and CHOP, specific markers of ER stress, while p53 expression, a specific marker for DNA damage, was unaffected. Conversely,

**Fig. 2.** Oxidative phosphorylation-dependent Bax activation in Rat-1 cells triggered by endoplasmic reticulum stress and DNA damage. **A)** Time kinetics of cell death and Bax activation. Rat-1 cells treated with 70  $\mu$ M tunicamycin (left panels) or 20 J/m<sup>2</sup> UV (right panels) were assayed for cell death (upper panels) and Bax dimerization (lower panels) at the indicated time points after treatment. The data points in the graphs represent the mean values of three independent experiments, and error bars correspond to 95% confidence intervals. **B)** Effect of suppression of oxidative phosphorylation on Bax dimerization. Cells were treated without or with 70  $\mu$ M tunicamycin (upper panel) or 20 J/m<sup>2</sup> UV (lower panel) in the absence or presence of the indicated inhibitors. The cells were harvested and analyzed for Bax oligomerization 15 and 12 hours after the tunicamycin and UV treatments, respectively. The concentrations of inhibitors used in this figure were as follows: rotenone, 0.1  $\mu$ M; antimycin A, 7  $\mu$ g/mL; oligomycin, 3  $\mu$ g/mL; Trolox, 0.6  $\mu$ M; Boc-Asp-fmk (BAF), 150  $\mu$ M. **C)** Effect of aurovertin B, a specific inhibitor of F<sub>1</sub>-adenosine triphosphatase on Bax dimerization. Rat-1 cells, either left untreated (control) or treated with 20 J/m<sup>2</sup> UV in the presence of the different concentrations of aurovertin B (20, 10, and 5  $\mu$ M), oligomycin (3  $\mu$ g/mL), or control vehicle for aurovertin B (chloroform, 0.1%), were subjected to Bax oligomerization assay 12 hours after UV treatment. **D)** Effect of suppression of oxidative phosphorylation on conformational change of Bax. Rat-1 cells were treated as in (B) and then subjected to immunoprecipitation analysis of Bax conformational change using conformation-specific anti-Bax mouse monoclonal antibody (6A7) followed by immunoblotting with an anti-Bax rabbit polyclonal antibody (N-20).



exposure of Rat-1 cells to UV caused increased expression of p53, but it did not change the expression of Bip and CHOP (Supplementary Fig. 3, available at: <http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue20>). We also observed that overexpression of a mutant Bcl-2 (Bcl-cb5), which contains a signal sequence that targets it to the ER, reduced cell death in response to tunicamycin treatment but not cell death caused by UV (Supplementary Fig. 1, A; available at: <http://jncicancer.spectrum.oxfordjournals.org/jnci/content/vol98/issue20>) (8). Collectively, these results suggested that oxidative phosphorylation, but not reactive oxygen species, plays an important role in Bcl-2-sensitive cell death induced by two kinds of cellular stress (ER stress and DNA damage) that activate disparate cell-signaling pathways.

### Essential Role for Oxidative Phosphorylation in Bax Activation by ER Stress and DNA Damage

Next, we sought to determine whether Bax was regulated by oxidative phosphorylation. We detected Bax activation by cross-linking the dimeric forms of the protein, before electrophoresis and immunoblotting, or by probing for the active conformer with a monoclonal antibody (6A7) that recognizes the amino terminal domain, which is exposed upon activation.

ER stress (tunicamycin, 70  $\mu$ M) and DNA damage (UV, 20 J/m<sup>2</sup>) caused the appearance of two bands of lower mobility than monomeric Bax in Rat-1 cells in parallel with the appearance of dead cells (Fig. 2, A). The uppermost of the bands in Fig. 2, A corresponds to a heterodimer of Bax and an uncharacterized protein,

and the one just below it represents the Bax homodimer (9,11). The appearance of the dimeric forms of Bax, observed at 15 and 12 hours after tunicamycin and UV treatments, respectively, was prevented by 7 µg/mL antimycin A and 3 µg/mL oligomycin and partially blocked by 0.1 µM rotenone (Fig. 2, B). Aurovertin B, which binds specifically to the F<sub>1</sub> subunit of the F<sub>0</sub>F<sub>1</sub>-ATPase and therefore inhibits the ATPase by a different mechanism from that of oligomycin, also reduced the amount of Bax dimers that were formed at 12 hours after UV treatment when added to cells at concentrations of 10 or 20 µM (Fig. 2, C). Treatment of Rat-1 cells with tunicamycin or UV also caused the appearance of an immunoreactive band on blots probed with the monoclonal antibody (6A7) specific for the active conformation of Bax (Fig. 2, D). The appearance of the band corresponding to activated Bax was prevented completely by antimycin A and oligomycin and partially prevented by rotenone (Fig. 2, D). Treatment of cells with either the antioxidant Trolox or the pan-caspase inhibitor BAF had no effect on the amount of Bax dimers or active conformer detected on immunoblots after exposure of Rat-1 cells to tunicamycin or UV (Fig. 2, B and D).

By means of the luciferin–luciferase reaction, we measured the intracellular ATP content of Rat-1 cells that had been treated for 12 hours with each of the inhibitors that were used to analyze Bax activation. For these experiments, all inhibitors were added at concentrations identical to those used to assay their effect on Bax activation in response to tunicamycin or UV exposure. The relative potency of the compounds in decreasing cellular ATP content matched their relative potency as inhibitors of Bax activation (Supplementary Fig. 4, available at: <http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue20>). The fact that rotenone was less effective at decreasing intracellular ATP levels might be because electron flow from respiratory chain complex II to complex III could continue uninterrupted even when complex I is inhibited. These results suggested that oxidative phosphorylation—specifically the activity of the F<sub>0</sub>F<sub>1</sub>-ATPase but not reactive oxygen species levels or caspase activity—is essential for the ER stress- and DNA damage-induced activation of Bax.

Oncogene activation promotes programmed cell death, and the circumvention of oncogene-dependent cellular suicide is regarded as a critical step in multistep carcinogenesis (12). Given the important role of the Bcl-2 protein family in oncogenesis as well as in programmed cell death regulation, we asked whether oxidative phosphorylation has a similar role in oncogene-dependent Bax activation and cell death. Overexpression of c-Myc or a constitutively active Ras protein (RasV12) (by transfection of vectors expressing these proteins into Rat-1 cells) increased Bax activation (as evidenced by the level of dimeric protein) and cell death in response to tunicamycin (40 µM) and UV (10 J/m<sup>2</sup>) compared to that observed when cells were treated with the same stresses in the absence of oncogene expression (Supplementary Fig. 5, available at: <http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue20>). The oncogene-dependent promotion of Bax activation and cell death was cancelled when cells were treated with antimycin A or oligomycin (Supplementary Fig. 5, available at: <http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue20>), suggesting that oxidative phosphorylation plays a role also in oncogene-dependent Bax activation and cell death.

We next asked whether suppression of Bax activation (i.e., dimerization and conformational change) via inhibition of oxidative phosphorylation prevents mitochondrial membrane permeabilization, the immediate functional consequence of Bax activity. We measured mitochondrial permeabilization by probing for

cytochrome c in cytosolic fractions of the cells (obtained by differential centrifugation) using a monoclonal antibody. Tunicamycin treatment of Rat-1 cells for 12 hours caused the appearance of cytochrome c in cytosolic fractions (Supplementary Fig. 6, available at: <http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue20>). This was efficiently prevented by treatment of the cells with antimycin A or oligomycin. These results indicate that oxidative phosphorylation plays an essential role in the mitochondrial membrane permeabilization that is mediated by activated Bax.

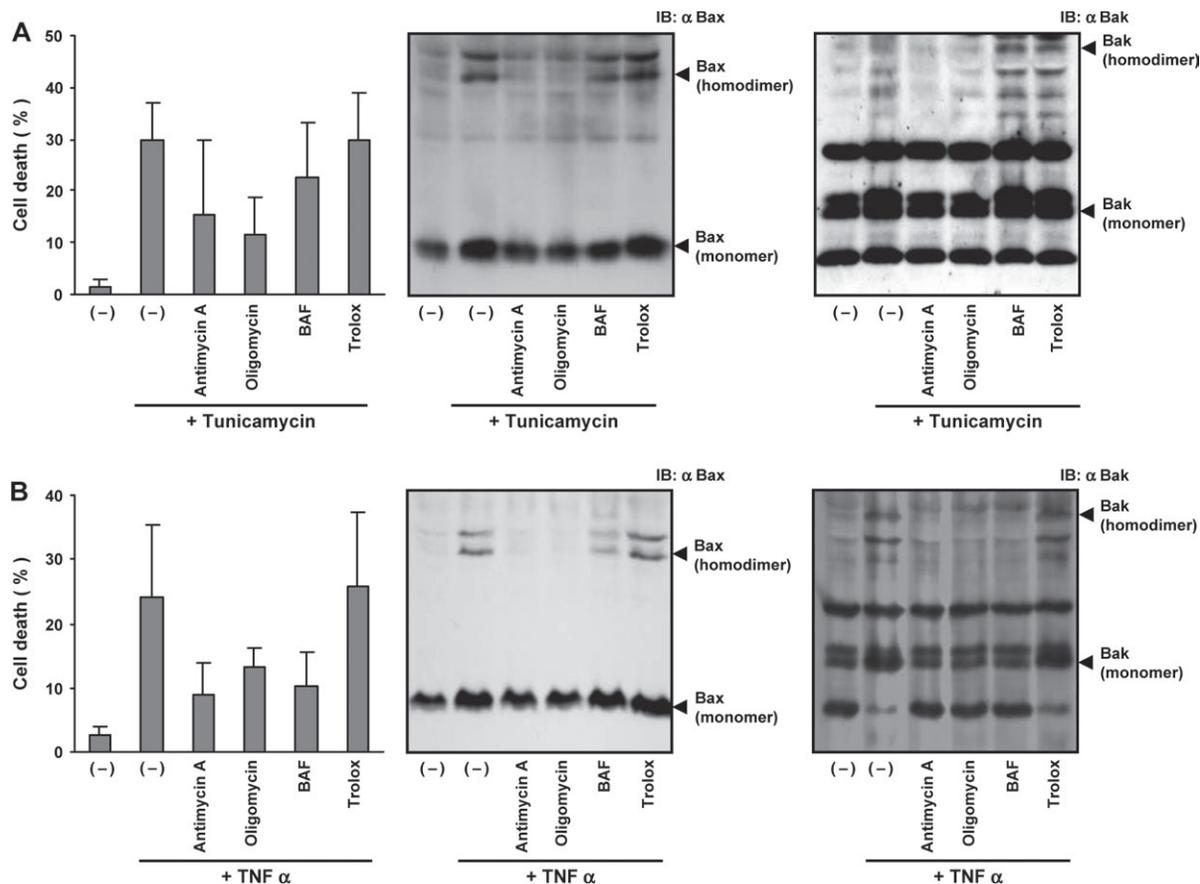
### **Oxidative Phosphorylation–Dependent Activation of Bax and Bak in Human Cancer Cells**

We wished to determine whether oxidative phosphorylation played a role in Bax activation in human epithelial cancer cells that was similar to the one we observed in rodent fibroblasts. Treatment of MCF-7 breast cancer cells with 90 µM tunicamycin (for 9 hours) or with 15 ng/mL TNF-α (for 6 hours) induced death of MCF-7 cells accompanied by Bax activation as evidenced by the appearance of protein dimers.

Both effects were reduced when oxidative phosphorylation was inhibited with 0.6 µg/mL antimycin A or 3 µg/mL oligomycin (Fig. 3, A and B, middle panels). We also observed in this study that tunicamycin and TNF-α consistently induce the appearance of a band that migrates as would be expected for a Bak homodimer (i.e., as a protein whose molecular weight equals approximately 48 kDa), indicating that Bak is activated and oligomerized by these death stimuli. We therefore examined the role of oxidative phosphorylation in Bak activation. Bak dimerization (observed 9 and 6 hours after treatment with tunicamycin and TNF-α, respectively) was inhibited by antimycin A and oligomycin, suggesting that Bak activation is also dependent on oxidative phosphorylation (Fig. 3, A and B, right panels). These inhibitors also reduced MCF-7 cell death in response to ER stress and TNF-α (Fig. 3, A and B, left panels). The percentage of tunicamycin-induced MCF-7 cell death at 9 hours (29.2%, 95% CI = 21.6% to 36.8%) was reduced to 15.3% (95% CI = 0.8% to 29.8%) by antimycin A and to 11.5% (95% CI = 3.9% to 19.1%) by oligomycin. TNF-α-induced MCF-7 cell death at 6 hours (24.0%, 95% CI = 12.6% to 35.4%) was reduced to 8.9% (95% CI = 3.9% to 13.9%) by antimycin A and to 13.3% (95% CI = 10.4% to 16.2%) by oligomycin. We also observed that Bax activation and death of HepG2 hepatocellular carcinoma cells (these cells do not express Bak) induced by UV or etoposide were similarly suppressed by inhibiting oxidative phosphorylation (Supplementary Fig. 7, available at: <http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue20>). Thus, oxidative phosphorylation is involved in Bax and Bak activation in human cancer cells.

### **Increased Glycolytic ATP Production Cannot Substitute Oxidative Phosphorylation in Bax and Bak Activation**

Next we attempted to determine whether the reduction in cell death and Bax and Bak activation that occurs when oxidative phosphorylation is inhibited is due to reduced availability of cellular ATP. To address this issue, we treated cells in such a manner as to decrease cellular ATP levels without impairing oxidative phosphorylation and examined the effects of this treatment on the extent of Bax and Bak activation in response to treatment with tunicamycin. To inhibit anaerobic ATP production by glycolysis,



**Fig. 3.** Oxidative phosphorylation-dependent activation of Bax and Bak and cell death of MCF-7 human breast cancer cells. **A** and **B**) MCF-7 breast cancer cells were treated without or with 90  $\mu$ M tunicamycin for 9 hours (**A**) or with 15 ng/mL tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) for 6 hours (**B**) in the absence or presence of the indicated inhibitors (antimycin A, 0.6  $\mu$ g/mL; oligomycin, 3  $\mu$ g/mL; Boc-Asp-

fmk [BAF], 150  $\mu$ M; Trolox, 0.6  $\mu$ M) and then assayed for cell death (**left panels**) as well as for Bax (**middle panels**) and Bak (**right panels**) oligomerization. In (**A**) and (**B**), the data in the graphs represent the mean values of three independent experiments, and **error bars** correspond to the upper 95% confidence intervals.

MCF-7 cells were cultured in the presence of 20 mM 2-deoxy-D-glucose (an inhibitor of glycolysis) and 1 mM pyruvate (to supplement substrates for oxidative phosphorylation). We confirmed that the addition of 20 mM 2-deoxy-D-glucose (and the resulting increase in osmolarity caused by addition) did not have a substantial effect on cellular viability (data not shown). Culture of MCF-7 cells for 12 hours in media supplemented with 20 mM 2-deoxy-D-glucose reduced cellular ATP to 69.3% (95% CI = 62.1% to 76.5%) of control (i.e., the level of ATP observed when cells were cultured in media containing 25 mM glucose). By comparison, culturing of the cells in media containing 25 mM glucose and 0.6  $\mu$ g/mL antimycin or 3  $\mu$ g/mL oligomycin reduced cellular ATP levels to 62.0% (95% CI = 46.9% to 77.1%) or to 78.5% (95% CI = 55.7% to 101.3%) of control, respectively (Fig. 4, A). Under these metabolic conditions, tunicamycin-induced Bax and Bak activation was not at all affected by 2-deoxy-D-glucose, but it was efficiently suppressed by antimycin A and oligomycin (Fig. 4, C, left panels). Consistently, the percentage of tunicamycin-induced MCF-7 cell death in culture medium containing 25 mM glucose (14.2%, 95% CI = 7.6% to 20.8%) was reduced to 4.6% (95% CI = 0.8% to 8.4%) by antimycin A and to 5.3% (95% CI = 1.0% to 9.6%) by oligomycin, but not at all by 2-deoxy-D-glucose, in the presence of which the percentage of cell death was 16.6% (95% CI = 11.4% to 21.8%) (Fig. 4, B).

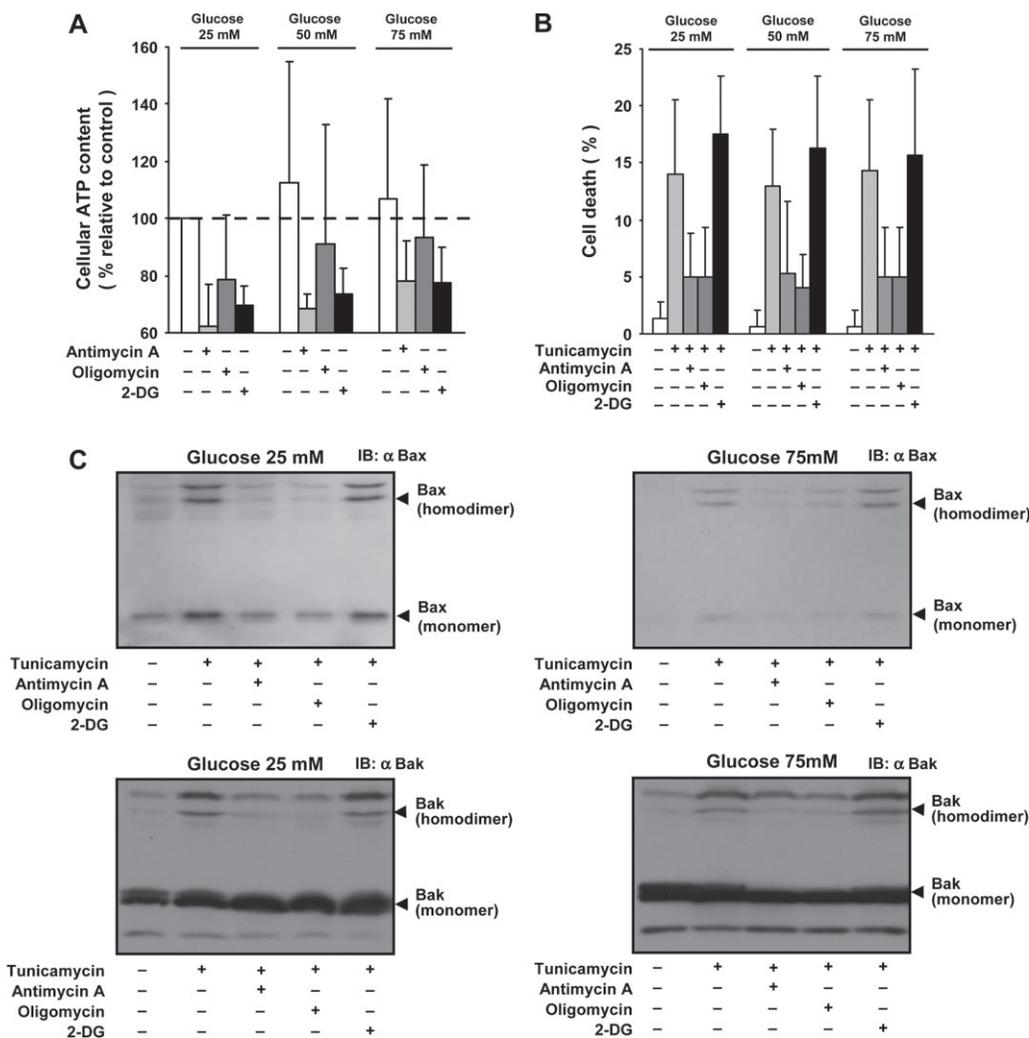
To further assess whether the effects of impaired oxidative phosphorylation on Bax and Bak activation were mediated by de-

creased cellular ATP, we increased the glucose concentration of the culture medium to raise ATP levels (by enhanced glycolytic ATP production) under conditions where oxidative phosphorylation was impaired by antimycin or oligomycin treatment. When glucose concentration in the medium was increased from 25 to 75 mM, the ATP levels in oligomycin (3  $\mu$ g/mL)-treated cells increased from 78.5% (95% CI = 55.7% to 101.3%) to 93.3% (95% CI = 68.0% to 118.6%) of the level observed in cells cultured in 25 mM glucose in the absence of oligomycin (Fig. 4, A). However, inhibition by antimycin A and oligomycin of Bax and Bak activation (Fig. 4, C) and cell death (Fig. 4, B) was not affected. These results suggest that a reduction in total cellular ATP levels per se was neither sufficient nor required to prevent Bax and Bak activation and cell death in response to tunicamycin treatment and therefore an additional consequence of impaired oxidative phosphorylation prevented Bax activation and cell death.

#### Increase of Oxidative Phosphorylation Preceding Bax and Bak Activation

Having established the essential role of oxidative phosphorylation in Bax and Bak activation in the cell death models used in this study, we next addressed whether oxidative phosphorylation is activated or not by cell death stimuli that activate Bax and Bak. Given the previous report demonstrating an increase in cellular ATP level preceding TNF- $\alpha$ -induced L929 cell death (13), we

**Fig. 4.** Lack of role for glycolytic adenosine triphosphate (ATP) production in the activation of Bax and Bak and cell death of MCF-7 breast cancer cells. **A)** MCF-7 cells cultured in the standard medium were switched to culture media containing 25, 50, and 75 mM glucose. After cells were cultured in the media for 3 hours, cells were treated with the indicated inhibitors (antimycin A, 0.6  $\mu\text{g}/\text{mL}$ ; oligomycin, 3  $\mu\text{g}/\text{mL}$ ; 2-deoxy-D-glucose [2-DG], 20 mM) for 12 hours and subjected to determination of cellular ATP content. The graph indicates ATP content per cell normalized to that of cells cultured in the presence of 25 mM glucose without inhibitors, which was set to 100%. The data represent the mean values of three independent experiments, and **error bars** correspond to the upper 95% confidence intervals. **B)** MCF-7 cells were treated as in (A), except that cells were treated without or with 90  $\mu\text{M}$  tunicamycin together with the inhibitors where indicated, and cell death was assayed 15 hours after tunicamycin treatment. The data represent the mean values of three independent experiments, and **error bars** correspond to the upper 95% confidence intervals. **C)** MCF-7 cells treated as in (B) were harvested 12 hours after tunicamycin treatment and subjected to in vitro cross-linking assay followed by immunoblot (IB) analysis to detect Bax (**upper panels**) and Bak (**lower panels**) dimers. The results of experiments done in the presence of 25 mM (**left panels**) and 75 mM (**right panels**) glucose are shown.



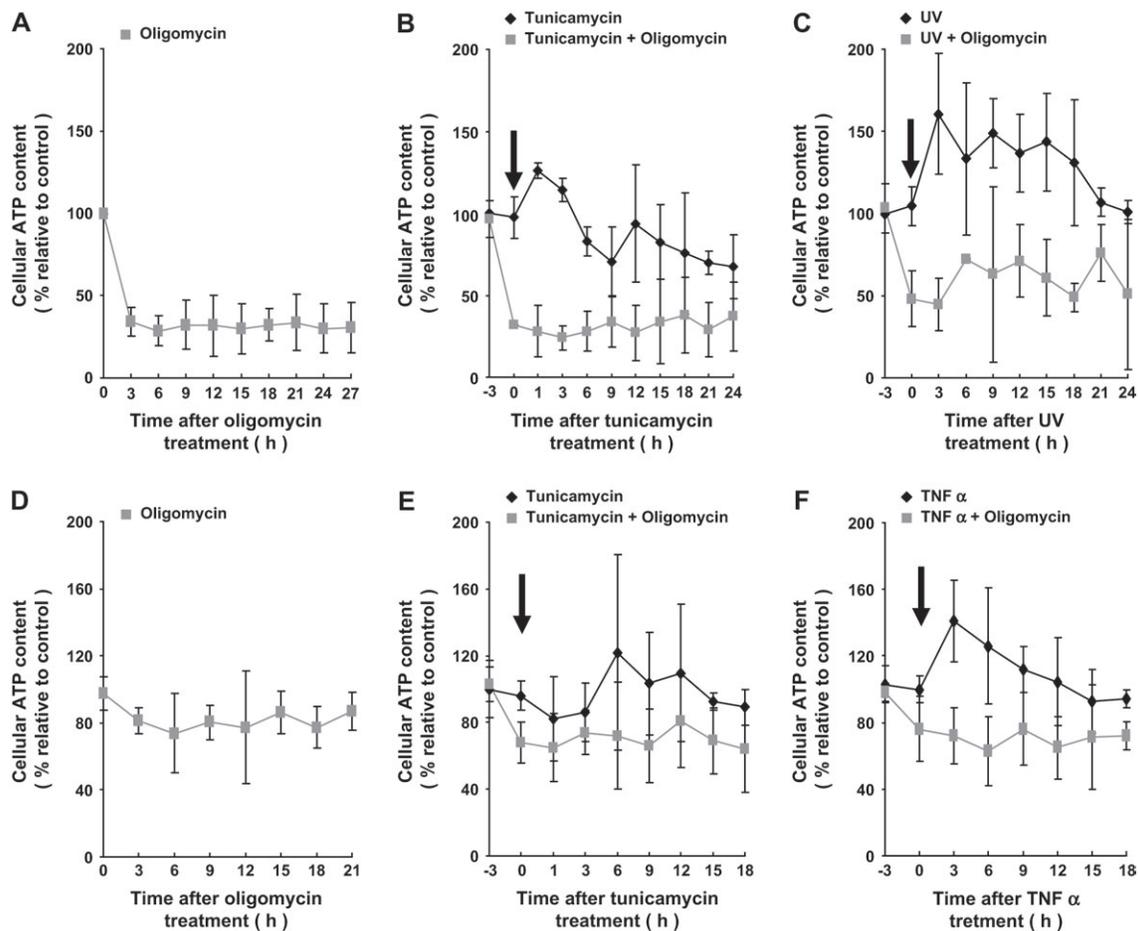
conducted a detailed time course analysis of cellular ATP levels after addition of cell death stimuli (Fig. 5) to detect any increase in oxidative phosphorylation before Bax and Bak activation and cell death. In this analysis, we measured ATP levels after exclusion of overtly dead cells because cellular ATP levels decline rapidly once cells undergo cell death. In Rat-1 cells treated with 70  $\mu\text{M}$  tunicamycin, the level of cellular ATP began to increase within 1 hour after tunicamycin treatment (Fig. 5, B). This increase, which is expressed as the percentage of the level observed in untreated cells harvested at each time point, preceded Bax activation and cell death, which started approximately 6 hours after treatment (Fig. 2, A). The increase in ATP level was abolished when cells were concomitantly treated with 3  $\mu\text{g}/\text{mL}$  oligomycin to inhibit oxidative phosphorylation (and hence Bax activation and cell death) (Fig. 5, B). Similarly, an oligomycin (3  $\mu\text{g}/\text{mL}$ )-sensitive increase in cellular ATP was detected approximately 3 hours after treatment of Rat-1 cells with 20  $\text{J}/\text{m}^2$  UV (Fig. 5, C). This increase also preceded Bax activation and cell death (Fig. 2, A). Furthermore, in MCF-7 cells, tunicamycin (90  $\mu\text{M}$ ) and TNF- $\alpha$  (15  $\text{ng}/\text{mL}$ ) induced an oligomycin-sensitive increase in cellular ATP level that preceded or closely paralleled Bax and Bak activation and cell death (Fig. 5, E and F, and not shown). These results suggest that increased oxidative phosphorylation is responsible for the increase in ATP level that we observe. The time course of the increase in oxidative phosphorylation relative

to that of Bax or Bak activation and cell death is consistent with the possibility that increased oxidative phosphorylation plays an important role in cell death–signaling pathways.

## DISCUSSION

In this study, we have provided evidence suggesting that oxidative phosphorylation, and possibly increased oxidative phosphorylation, plays a crucial role in Bax and Bak activation and cell death. The requirement for oxidative phosphorylation was observed in several different cell types (including human cancer cells) stimulated by disparate stimuli. Furthermore, we observed that increased ATP production by anaerobic glycolysis cannot substitute for oxidative phosphorylation in promoting Bax and Bak activation and cell death.

Although this is, to our knowledge, the first report to propose such a specific role for oxidative phosphorylation in Bax and Bak activation and cell death, our findings are consistent with previous work that suggested a role for oxidative phosphorylation in Bax-induced cell death. Some of the work was done in yeast, where it was shown that oxidative phosphorylation was essential for cell death induced by transgenic expression of Bax (14,15) and that cells become resistant to Bax-induced cell death under fermentative conditions. Transgene-mediated Bax expression



**Fig. 5.** Oxidative phosphorylation–dependent increase of cellular adenosine triphosphate (ATP) level preceding Bax and Bak activation. Rat-1 fibroblasts (A–C) and MCF-7 breast cancer cells (D–F) were treated with oligomycin (3 μg/mL) alone (A and D) or with tunicamycin (70 μM in B and 90 μM in E), 20 J/m<sup>2</sup> UV (C), and 15 ng/mL tumor necrosis factor-α (TNF-α) (F) in the absence and presence of oligomycin (3 μg/mL). Live (adherent) cells were selectively collected and subjected to measurement of cellular ATP content at the indicated

time points. Untreated control cells cultured along with the treated cells were also harvested at each time point, and their ATP contents (set to 100%) were used for normalization. The data points represent the mean values of three independent experiments, and **error bars** correspond to 95% confidence intervals. **Arrows** in the graphs indicate the time points at which cells were treated with the death stimuli (oligomycin was added 3 hours before death stimuli).

selected for yeast cells with the *petite* phenotype (these cells are unable to carry out oxidative phosphorylation), implying that decreased oxidative phosphorylation may be an adaptive mechanism to survive Bax expression (15).

Additional support for the idea that oxidative phosphorylation plays a critical role in Bax and Bak activation and cell death comes from experiments that showed that the mitochondrial genome, which encodes protein components that are essential for oxidative phosphorylation, is required for various types of cell death (16,17, and references therein). Furthermore, the essential role of the F<sub>0</sub>F<sub>1</sub>-ATPase in Bax-induced cell death has been demonstrated not only in yeast cells but also in mammalian cells using oligomycin (18), and oligomycin has been shown to inhibit calphostin c-induced Bax oligomerization in human glioma cells (19). Antimycin A was not effective in these experiments. However, aside from its activity as an inhibitor of oxidative phosphorylation, antimycin A can mimic a cell death–inducing BH3 domain by interacting directly with Bcl-2 family proteins (20). We observed that concentrations of antimycin A that were higher than those needed to inhibit respiration induce Bax activation even when oxidative phosphorylation was inhibited (Serizawa S and Kitanaka C: unpublished observation). Thus, the opposing

actions of antimycin A may obscure its effect on Bax and Bak activation under some circumstances, and negative results obtained from experiments using antimycin A should be interpreted with caution.

Another important observation in support of our finding that oxidative phosphorylation is essential for Bax and Bak activation and cell death is that Bcl-2, which prevents Bax and Bak activation without interacting directly with these proteins (1,2), inhibits mitochondrial respiration and complex I activity (21,22). Although Bcl-2 can inhibit Bax and Bak activation via sequestration of BH3-only proteins (1,2), inhibition of oxidative phosphorylation may be another mechanism by which Bcl-2 inhibits Bax and Bak activation. Thus, our results and previous work strongly suggest that oxidative phosphorylation is involved in a fundamental mechanism of Bax and Bak activation and cell death that is common to many cell types. However, this does not imply that oxidative phosphorylation is required for cell death mediated by Bcl-2 proteins in all cases (23,24).

Why does oxidative phosphorylation, but not the production of ATP by glycolysis, contribute to Bax and Bak activation? One possibility is that oxidative phosphorylation and glycolysis, while similar in terms of ATP production, may differentially modulate

the intracellular level of adenosine diphosphate and/or adenosine monophosphate (AMP), the latter of which activates the AMP-activated protein kinase pathway that was recently shown to play a role in Bax activation (25,26). Another possibility is that the regulation of Bax and Bak by oxidative phosphorylation takes place in the mitochondrial compartment. We observed that cell death in Rat-1 cells treated with tunicamycin or UV was accompanied by minimal translocation of Bax from the cytosol to the mitochondria (Supplementary Fig. 8, available at: <http://jncicancer.spectrum.oxfordjournals.org/jnci/content/vol98/issue20>) and that a large fraction of Bax was already present in mitochondria even in untreated, healthy cells. In contrast to what occurs in Rat-1 cells, Bax does translocate to mitochondria upon tunicamycin treatment in MCF-7 cells, and translocation can be blocked by inhibition of oxidative phosphorylation (Tomiya A and Kitanaka C: unpublished data). Nonetheless, activation (dimerization) of Bax and Bak was inhibited with similar efficiency in both cell types by inhibition of oxidative phosphorylation (Figs. 3 and 4), despite the fact that Bak is an integral mitochondrial protein. Therefore, these findings strongly suggest that cytosol to mitochondria translocation is not the step where oxidative phosphorylation and the processes of Bax and Bak activation interact and that the interaction between oxidative phosphorylation and the activation of Bax and Bak takes place within mitochondria. Mitochondrial matrix ATP produced by oxidative phosphorylation (but not cytosolic ATP) has a specific role in the regulation of the permeability transition pore of the inner mitochondrial membrane, and inhibition of oxidative phosphorylation blocks opening of the pore (27,28). Therefore, one possible mechanism by which oxidative phosphorylation could control Bax activation is the induction of the permeability transition, which has been suggested to be essential for Bax activation (29,30). Since Bax and Bak are physically associated with the permeability transition pore complex (31,32), one attractive hypothesis would be that the conformational change of the pore components taking place during the permeability transition promotes conformational change of Bax and Bak to their active states.

The possibility that cells could functionally inactivate Bax and Bak by switching energy metabolism from aerobic oxidative phosphorylation to anaerobic glycolysis has important implications for tumor biology. It is well known that tumor cells depend largely on glycolysis for ATP production (33). In solid tumors, cells often grow faster than an adequate network of blood vessels can be formed, and tumor hypoxia as a result of insufficient blood supply is believed to cause tumor cells to switch to anaerobic metabolism (34–37). However, it is important to note that although anaerobic glycolysis is apparently disadvantageous in terms of energy production compared to oxidative metabolism, tumor cells tend to carry out anaerobic glycolysis even in the presence of sufficient oxygen, a phenomenon known as the Warburg effect (38). In addition, it has been demonstrated *in vitro* that cervical cancer–derived, non-neoplastic stromal cells (fibroblasts) respond to hypoxia and produce angiogenic factors better than cervical cancer cells themselves (39). Furthermore, an *in vivo* study utilizing transgenic mice expressing green fluorescent protein under the control of the hypoxia-sensitive vascular endothelial growth factor (VEGF) gene promoter revealed that it is not tumor cells but nontransformed stromal cells (fibroblasts) that show high VEGF promoter activity within spontaneously formed mammary tumors (40). These findings suggest that tumor cells “choose” to carry out anaerobic metabolism instead of

“being forced” to do so and may therefore be in much less need of oxygen than normal cells. To explain this phenomenon, we propose that shifting metabolism from oxidative phosphorylation to glycolytic ATP production is a means by which tumor cells protect themselves from programmed cell death. This idea is supported by a recent report suggesting that enhanced glucose metabolism has a critical role in Akt-mediated Bax inactivation (41). A means to survive programmed cell death is especially critical for tumor cells because they must withstand stresses caused by oncogenic genetic changes as well as by other stresses that normal cells would not encounter (12). Given the central role of Bax and Bak in programmed cell death, this could be efficiently achieved by inactivating Bax and Bak. Indeed, inactivation of Bax alone (5) or of both Bax and Bak has been shown to confer a survival advantage and be selected for during tumorigenesis and tumor progression *in vivo* (6). In human tumors, inactivation of Bax and Bak by mutation and impaired expression (in addition to overexpression of Bcl-2–like pro-survival proteins and inactivation of BH3-only members of the Bcl-2 family) has been reported (7), and our results suggest that anaerobic metabolism is another means by which Bax and Bak can be inactivated.

Tumor hypoxia in solid tumors has been associated with poor prognosis and therapy resistance (35,36,42). Previously, hypoxia was considered to contribute to increased resistance against cell death–inducing therapies and hence poor prognosis by promoting tumor cell death and causing only those tumor cells that are resistant to programmed death to survive and predominate in the tumor mass (36,42). However, recent *in vitro* observations suggest that hypoxia rather protects tumor cells from programmed death particularly when the tumor cells are maintained at non-acidic pH (43–45). Furthermore, magnetic resonance spectroscopic analysis demonstrated that the intracellular pH is indeed maintained nonacidic in tumor cells *in vivo* (46). In conjunction with our idea that oxidative phosphorylation is essential for Bax and Bak activation, these observations suggest the possibility that hypoxia may confer a survival advantage to tumor cells through inhibition of oxidative phosphorylation and consequently of Bax and Bak activation. It would be worthwhile to examine the effect of hypoxia on the activation of Bax and Bak. However, as discussed above, tumor cells have the ability to shift the metabolism from aerobic oxidative phosphorylation to anaerobic glycolysis via, for instance, oncogene activation even in the presence of adequate oxygen supply (34). This being the case, the actual role of hypoxia in the switch to predominantly glycolytic metabolism by tumor cells often observed *in vivo* is unclear and may be less important than has been generally believed, in that tumor hypoxia could occur secondarily as a consequence of decreased requirement for oxygen.

As for their implications for cancer treatment, our data suggest that changing the tumor metabolism from anaerobic glycolysis to oxidative phosphorylation may restore the cells’ sensitivity to programmed cell death and thus enhance the effect of conventional antitumor modalities such as radiotherapy and chemotherapy. Given the critical role of hypoxia-inducible factor in activating glycolytic metabolism (34), targeting this transcription factor may be a rational approach for inducing this metabolic change. It is interesting to note that caffeine, known to stimulate oxidative phosphorylation (47), has been reported to induce conformational change of overexpressed Bax to an active form and cell death in human cancer cells (48). Other factors that control

oxidative phosphorylation, e.g., intramitochondrial calcium (49), could be potential therapeutic targets. The role of intramitochondrial calcium in the increase in oxidative phosphorylation in response to activation of the cell death pathway that we observed needs to be investigated.

Although we have shown for the first time in this study that oxidative phosphorylation plays a critical role in the activation of Bax and Bak, there are also limitations to this study. First, it remains unknown to what extent the principal finding of this study (oxidative phosphorylation-dependent activation of Bax and Bak) could be generalized to cell types other than those examined in this study. Second, although we used a variety of inhibitors of the respiratory chain and oxidative phosphorylation, each acting by distinct mechanisms, the pharmacologic inhibition of the respiratory chain and oxidative phosphorylation may not faithfully recapitulate the conditions of decreased oxidative phosphorylation taking place in tumor cells. Thus, further studies will be required to examine the relevance of our findings to *in vivo* tumors.

In the 1920s, Otto Warburg shed the first light on the unique energy metabolism of cancer cells (38). His original proposal that “defective” oxidative metabolism has a causal role in tumorigenesis and in the glycolytic metabolism of cancer cells has not been validated (34). However, our results suggesting the involvement of oxidative phosphorylation in programmed cell death reilluminate from a different perspective the unique metabolism of tumor cells as a critical factor in tumorigenesis and as an attractive target in cancer therapy.

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## NOTES

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