

Reversal of Physiological Stress-Induced Resistance to Topoisomerase II Inhibitors Using an Inducible Phosphorylation Site-Deficient Mutant of I κ B α

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Received March 2, 2001; accepted May 22, 2001

This paper is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

Physiological stress conditions associated with the tumor microenvironment play a role in resistance to anticancer therapy. In this study, treatment of EMT6 mouse mammary tumor cells with hypoxia or the chemical stress agents brefeldin A (BFA) or okadaic acid (OA) causes the development of resistance to the topoisomerase II inhibitor etoposide. The mechanism of physiological stress-induced drug resistance may involve the activation of stress-responsive proteins and transcription factors. Our previous work shows that treatment with BFA or OA causes activation of the nuclear transcription factor NF- κ B. Pretreatment with the proteasome inhibitor carbobenzyloxyl-leuciny-leuciny-leucinal inhibits stress-induced NF- κ B activation and

reverses BFA-induced drug resistance. To test whether NF- κ B specifically mediates stress-induced drug resistance, an inducible phosphorylation site-deficient mutant of I κ B α (I κ B α M, S32/36A) was introduced into EMT6 cells. In this study, we show that I κ B α M expression inhibits stress-induced NF- κ B activation and prevents BFA-, hypoxia-, and OA-induced resistance to etoposide. These results indicate that NF- κ B activation mediates both chemical and physiological drug resistance to etoposide. Furthermore, they imply that coadministration of agents that inhibit NF- κ B may enhance the efficacy of topoisomerase II inhibitors in clinical cancer chemotherapy.

Solid tumors often have irregular and inadequate vascularization because of the uncontrolled cellular growth associated with tumor formation. Inadequate blood flow creates cell subpopulations within tumors that are hypoxic and/or glucose-deprived (Vaupel et al., 1989). These physiological stress conditions can result in tumor subpopulations with altered biochemical properties. Alterations such as decreased growth fraction or enhanced DNA repair can result in the development of intrinsic resistance against topoisomerase II-directed anticancer agents (Shen et al., 1987). Resistance to topoisomerase II inhibitors can also be induced by chemical stress agents that cause the inhibition of protein glycosylation, release of intracellular calcium stores, or disruption of endoplasmic reticulum (ER)-to-Golgi transport (Hughes et al., 1989; Lin et al., 1998). Taken together, these results suggest that physiological-based chemotherapeutic resistance may involve the induction of cellular stress pathways.

Under chemical or physiological stress conditions, ER function is often compromised because of the accumulation of normally folded proteins in the ER (Pahl and Baeuerle, 1997). High ER protein levels activate a cellular stress pathway known as the ER-overload response (EOR). In this pathway, ER protein overload causes the release of intracellular Ca²⁺, formation of reactive oxygen intermediates, and activation of the nuclear transcription factor NF- κ B (Pahl and Baeuerle, 1997). Evidence now shows that physiological and chemical stress agents that result in drug resistance cause the activation of the EOR pathway and the transcription factor NF- κ B (Hughes et al., 1989; Pahl and Baeuerle, 1997; Lin et al., 1998). NF- κ B is a heterodimeric transcription factor usually composed of the p65 and p50 DNA-binding subunits (Urban et al., 1991). Under most circumstances, NF- κ B is in an inactive state, bound to an inhibitory protein, I κ B, in the cytosol. Three major isoforms of I κ B have been identified, of which I κ B α is believed to be the predominant form (Tran et al., 1997). To activate NF- κ B, I κ B α is phosphorylated, ubiquitinated, and then degraded by proteasomes (Henkel et al., 1993). Point-mutation analysis has shown that I κ B α is specifically phosphorylated at two resi-

This work was supported in part by Army Breast Cancer Initiative Award #99-1-9186 (to K.A.K.) and by a faculty research enhancement award from the George Washington University Medical Center (to K.A.K.).

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ABBREVIATIONS: ER, endoplasmic reticulum; EOR, endoplasmic reticulum-overload response; NF- κ B, nuclear factor- κ B; BFA, brefeldin A; OA, okadaic acid; MG-132, carbobenzyloxyl-leuciny-leuciny-leucinal; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; I κ B α M, S32/36A mutant inhibitory nuclear factor- κ B protein α ; VCT, vector lacking I κ B α M insert; TBST, Tris-buffered saline/Tween 20; BSA, bovine serum albumin; ANOVA, analysis of variance; UPR, unfolded protein response.

dues, serines 32 and 36, and phosphorylation site-deficient mutants are incapable of activating NF- κ B (DiDonato et al., 1996). Degradation of I κ B exposes a nuclear localization sequence that allows the translocation of NF- κ B into the nucleus, where it then binds to κ B motifs in promoter regions and directs the transcription of NF- κ B-sensitive genes (Hahaj and Sun, 1999).

In addition to its role in cellular stress responses, NF- κ B activation is known to protect cells from apoptosis. NF- κ B activation suppresses the activation of caspase-8 through the regulation of tumor necrosis factor receptor-associated factor protein and inhibitor of apoptosis protein (Wang et al., 1998) and prevents cytochrome *c* release through activation of A1/Bfl-1, a Bcl-2 family member (Wang et al., 1999). Inhibition of NF- κ B activation with expression of a mutant I κ B α sensitizes tumor cells to apoptotic death by tumor necrosis factor, paclitaxel, and daunorubicin (Wang et al., 1996; Batra et al., 1999; Huang et al., 2000). Mutant I κ B α expression in vivo significantly reduces growth of head and neck squamous cell carcinoma (Duffey et al., 1999) and sensitizes chemoresistant tumors to the toxic effects of camptothecin (Cusack et al., 2000). These results suggest that physiological stress-induced NF- κ B activation may modulate the expression of apoptosis genes and that inhibition of NF- κ B activation may prevent stress-induced drug resistance.

We have shown that EMT6 mouse mammary tumor cells treated with the chemical stress agents brefeldin A (BFA) or okadaic acid (OA) causes NF- κ B activation and resistance to the topoisomerase II inhibitor etoposide (Lin et al., 1998). Treatment with BFA disrupts protein transport from the ER to the Golgi apparatus and causes activation of the EOR response (Pahl and Baeuerle, 1997). OA treatment inhibits the PP1 and PP2A phosphatases, resulting in phosphorylation of I κ B and NF- κ B activation (Trevenin et al., 1990). We have also shown that pretreatment with the proteasome inhibitor MG-132 inhibits NF- κ B activation induced by BFA and reverses BFA-induced resistance to etoposide (Lin et al., 1998). In the present study, we show that BFA, hypoxia, and OA induce resistance to the clinically relevant topoisomerase II inhibitor etoposide. We tested whether specific inhibition of NF- κ B with the phosphorylation site-deficient mutant of I κ B α (I κ B α M, S32/36A) prevents stress-induced NF- κ B activation and reverses BFA, hypoxia, and OA-induced resistance to etoposide. Our results show that NF- κ B is a key mediator of both chemical and physiological resistance to etoposide.

Materials and Methods

Cell Culture. EMT6 mouse mammary tumor cells, provided by Dr. Sara Rockwell (Yale University, New Haven, CT), were grown in a monolayer in Waymouth's MB 752/1 medium with L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (Sigma, St. Louis, MO), 100 units/ml streptomycin, and 25 μ g/ml gentamicin sulfate (Biofluids, Rockville, MD). Cells were maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C and passaged every 3 to 4 days.

Reagents and Treatments. Brefeldin A (Sigma) was dissolved in 70% ethanol to a concentration of 10 mg/ml and stored at 4°C. For electrophoretic mobility shift assay (EMSA) and luciferase assays, cells were exposed to 10 μ g/ml BFA for 2 h and then incubated for 2 h in BFA-free media. For colony-forming assays, cells were exposed to BFA for 2 h and then incubated in BFA-free media for an additional

6 h. Okadaic acid (Calbiochem, La Jolla, CA) was kept at a concentration of 100 μ M in dimethyl sulfoxide and stored at -20°C. Cells were treated with 60 nM OA for 8 h in all experiments. Etoposide (Sigma) at 100 μ M in dimethyl sulfoxide was stored at -20°C. Cells were treated with 10 to 50 μ M etoposide 1 h before clonogenicity assay. Ponasterone A (Invitrogen) was rehydrated in 70% ethanol to a concentration of 10 mM and stored at -20°C. For all experiments, cells were treated with 10 μ M ponasterone A for 24 h to obtain maximal expression of I κ B α M. For all assays involving hypoxia, cells were grown in 75-cm² glass flasks for 48 h and then exposed to continuous hypoxia as described previously (Rockwell et al., 1982) for either 2 h (EMSA and luciferase assays) or 8 h (colony-forming assays). For EMSA and luciferase assays, the length of stress treatment was chosen to correspond with the time of maximal stress-induced NF- κ B activation as determined previously (Lin et al., 1998; data not shown).

Inducible I κ B α M Transfection. EMT6 cells were stably transfected with a phosphorylation site-deficient mutant of I κ B α (I κ B α M, S32/36A, provided by Dr. Michael Karin, University of California, San Diego), which also contains three hemagglutinin (HA) tags (DiDonato et al., 1996) or a control vector lacking I κ B α M (VCT) using the ecdysone-inducible expression system (Invitrogen). The I κ B α M gene was first ligated into the inducible plasmid pIND to create the pIND-I κ B α M plasmid. The other plasmid in the system, pVgRXR, encodes a modified ecdysone receptor and retinoid X receptor that dimerize in the presence of the inducing agent, ponasterone A, and binds response elements on the pIND plasmid. For transfection, EMT6 cells were seeded at a density of 3 to 4 \times 10⁴ cells/ml in 25-cm² flasks and were grown for 20 h. Cells were transfected for 1 h with 1 μ g of pIND or pIND-I κ B α M plasmid, 5 μ g of pVgRXR plasmid, and 36 μ l lipid transfection reagent (TransFast; Promega, Madison, WI) in 2.5 ml of serum-free Waymouth's media. Transfected cells were then seeded in 100 mm² tissue-culture dishes and treated with 400 μ g/ml hygromycin B (Invitrogen) to select for incorporation of the pIND vector. Selected clones were screened by Western blot and luciferase assay for inducible I κ B α M expression. Cell lines were maintained in Waymouth's media containing 400 μ g/ml hygromycin and grown in Waymouth's media without hygromycin for 40 h before experimentation.

Western Blot Analysis. Cells were seeded in 100-mm² dishes and grown for 18 h. After treatment with ponasterone A, total cell lysates were collected by homogenizing cell pellets in 100 μ l of 1 \times SDS sample buffer (125 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, and 0.006% bromophenol blue). Protein lysate (20–100 μ g) was mixed with 2 \times SDS (250 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 0.012% bromophenol blue, and 2% β -mercaptoethanol), separated on a SDS-polyacrylamide gel (4% stacking gel, pH 6.8; 10% resolving gel, pH 8.8; 30:0.8 acrylamide/bisacrylamide), and transferred to a nitrocellulose membrane by electrophoresis. After transfer, the membrane was blocked in 1 \times TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) with 1% bovine serum albumin (BSA) and then probed with an anti-I κ B α antibody (C21; Santa Cruz Biochemicals, Santa Cruz, CA) diluted 1:1000 in 1 \times TBST with 1% BSA overnight at 4°C. The membrane was then washed with 1 \times TBST and incubated with an horseradish peroxidase-conjugated IgG anti-rabbit secondary antibody (1:10,000 dilution in 1 \times TBST with 1% BSA) for 1 h at room temperature. Immunoreactive bands were observed with enhanced chemiluminescent reagent (Pierce Chemical, Rockford, IL). After observation, the membrane was incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 0.67% β -mercaptoethanol) at 50°C for 30 min, washed in 1 \times TBST for 1 h, and probed again with anti-HA and anti-actin primary antibodies (Santa Cruz Biochemicals).

Transient Transfection and Luciferase Reporter Gene Assay. We obtained a luciferase reporter plasmid, pTk-(κ B)₆-Luc (provided by Dr. Heike Pahl, University Hospital, Freiburg, Germany), that contains six NF- κ B binding sites (κ B elements) upstream of a minimal thymidine kinase promoter (Bachelier et al., 1991). Cells

were seeded at a density of 3 to 4×10^4 cells/ml in 60-mm² dishes or 25-cm² flasks and grown for 20 h. Cells were transfected with 2.5 ml of serum-free Waymouth's media containing 3 μ g of the luciferase reporter plasmid and 1 μ g of pcDNA3.1-*lacZ* (Invitrogen) in 12 μ l of reagent (TransFast; Promega). After drug treatments, cells were lysed for 15 min at room temperature in 400 μ l of reporter lysis buffer (Promega) and cleared of cell debris by centrifugation. For the luciferase assay, 100 μ l of luciferase assay reagent containing luciferol (Promega) was added to 20 μ l of cell lysate. Light emission was measured using a Beckman scintillation counter using the single-photon monitor mode over a 1-min interval. Cells were also assayed for *lacZ* expression to correct for differences in transfection efficiency. Cell lysate (100–150 μ l) was mixed with and equal amount of 2 \times assay buffer containing *o*-nitrophenyl- β -D-galactopyranoside (Promega) and incubated for 2 h at 37°C. Absorbance was measured at 420 nm was measured, and the relative β -galactosidase activity for each sample was used to normalize luciferase activities.

EMSA. EMSA was performed as described previously (Lin et al., 1998). Briefly, cells were seeded at a density of 3 to 4×10^4 cells/ml in 150-mm² dishes or 150-cm² glass flasks. After drug treatment, cells were lysed in 100 μ l of lysis buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride) for 5 min at 4°C. Cell nuclei were separated by centrifugation at 5000 rpm for 5 min at 4°C, washed with 500 μ l of washing buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM dithiothreitol, 0.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride), and broken by three freeze-thaw cycles. To construct the probe, 3.5 pmol of oligonucleotide containing the NF- κ B consensus sequence (Promega) was incubated with 1 μ l of [γ -³²P]ATP (10 mCi/ml, 6000 Ci/mmol; Amersham Pharmacia Biotech, Arlington Heights, IL), 5 units of T4 polynucleotide kinase (Promega), and 10 μ l of end-labeling buffer at 37°C for 1 h and then terminated with 90 μ l 1 \times Tris/NaCl/EDTA buffer (Sigma) and passed through a G-25 spin column (Worthington Biochemicals, Freehold, NJ). Nuclear protein extract (15–20 μ g) was incubated with 3 μ g of poly dI · dC and 0.035 pmol of radiolabeled oligonucleotide (100,000–200,000 cpm) in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl₂, 0.5 mM dithiothreitol, and 4% glycerol) at room temperature for 20 min and separated on a nondenaturing 6% polyacrylamide gel (30:1 acrylamide/bisacrylamide, 0.5 \times Tris/borate/EDTA, and 2.5% glycerol). The resulting gel was transferred to filter paper, dried under vacuum pressure, and exposed to X-ray film.

Colony-Forming Assay. Cells were seeded in 25-cm² plastic flasks or 75-cm² glass flasks at a density of 3 to 4×10^4 cells/ml and grown for 18 to 40 h before treatment. VCT and I κ B α M cells were treated with ponasterone A 24 h before colony assay, with stress 8 h before colony assay, and with 10 to 50 μ M etoposide 1 h before colony assay. After drug treatments, cells were harvested with trypsin and serially diluted in Waymouth's medium, as described previously (Lin et al., 1998). After 7 to 10 days, colonies were stained with 0.25% crystal violet and counted. For each treatment, the percentage of control cell survival was determined by dividing the cell survival of drug-treated cells by the cell survival of appropriate nontreated or solvent-treated cells.

Statistics. For colony-forming assays, the results shown are the average percentage of control survival \pm S.E.M. from three to five independent experiments, with three replicates per experiment. For luciferase assays, the results shown are the relative control luciferase activity \pm S.E.M. from three to five independent experiments. Statistically significant changes in these data were determined using one-way ANOVA with multiple comparisons performed using Bonferroni's test using $p < 0.05$ (Motulsky, 1995).

Results

Treatment with BFA, Hypoxia, and OA Induce Resistance to Etoposide. Chemical and physiological stress con-

ditions are known to activate ER stress pathways and induce resistance to topoisomerase II inhibitors (Hughes et al., 1989; Lin et al., 1998). We first determined the effect of the ER stress agents BFA, hypoxia, and OA on the clonogenic survival of etoposide-treated EMT6 cells. Cells were exposed to hypoxia for 8 h, 60 nM OA for 8 h, or 10 μ g/ml BFA for 2 h, followed by recovery in BFA-free media for 6 h. Etoposide at various concentrations was added during the last hour of stress treatment before analysis by colony-forming assay. Plating efficiencies were corrected for survival changes caused by treatment with BFA (plating efficiency = 110% of control), hypoxia (plating efficiency = 80% of control), or OA (plating efficiency = 38% of control). Figure 1 shows that pretreatment with BFA, hypoxia, or OA causes greatly enhanced cell survival in the presence of etoposide compared with nonstressed cells. These data suggest that the chemical and physiological conditions known to activate the EOR pathway induce resistance to etoposide.

Inducible I κ B α M Expression in EMT6 Cells. We and others have shown that chemical and physiological stress agents that cause ER stress lead to the activation of NF- κ B (Lin et al., 1998; Pahl and Baeuerle, 1997). Furthermore, inhibition of NF- κ B is known to enhance the toxicity of cancer chemotherapeutics (Wang et al., 1996; Batra et al., 1999; Cusack et al., 2000; Huang et al., 2000) and reverse stress-induced drug resistance (Lin et al., 1998). Therefore, we hypothesized that BFA, hypoxia, and OA cause resistance to topoisomerase II inhibitors through the activation of NF- κ B. To test this hypothesis, we selected EMT6 cells transfected with either a phosphorylation site-deficient mutant of I κ B α (I κ B α M) or a control vector lacking I κ B α M (VCT) using the ecdysone-inducible expression system. After selection in hygromycin, we screened transfectants by using Western blot analysis for expression of I κ B α M after treatment with the inducing agent, ponasterone A.

The I κ B α M we obtained also contains three HA tags (DiDonato et al., 1996), which cause the mutant protein to migrate more slowly than wild-type I κ B α when analyzed by gel electrophoresis. Figure 2 shows that both VCT cells treated with ponasterone A and noninduced I κ B α M cells

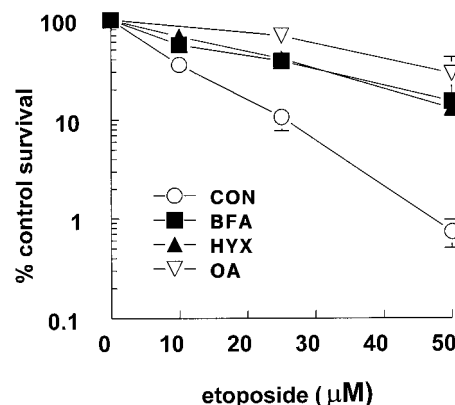


Fig. 1. Brefeldin A, hypoxia, and okadaic acid treatment cause resistance to etoposide toxicity. EMT6 cells were treated with either 10 μ g/ml brefeldin A (BFA) for 2 h followed by a BFA-free recovery for 6 h, hypoxia (HYX) for 8 h, or okadaic acid (OA) for 8 h. Etoposide (10, 25, or 50 μ M) was added during the last hour of stress treatment before analysis by colony-forming assay. The toxicity of etoposide in nonstressed cells (CON) is also shown. Results shown are the mean percentage of control cell survival \pm S.E.M. from three to five independent experiments.

express levels of wild-type $\text{I}\kappa\text{B}\alpha$ comparable with that of nontransfected EMT6 cells. However, $\text{I}\kappa\text{B}\alpha\text{M}$ cells treated with ponasterone A for 24 h express the slower-migrating $\text{I}\kappa\text{B}\alpha\text{M}$ only. We confirmed these findings by reprobing these blots with an anti-HA primary antibody (Fig. 2). Only ponasterone-induced $\text{I}\kappa\text{B}\alpha\text{M}$ cells express protein that is immunoreactive to the anti-HA antibody. The absence of wild-type $\text{I}\kappa\text{B}\alpha$ in extracts from induced $\text{I}\kappa\text{B}\alpha\text{M}$ cells may be explained by the rapid association and dissociation of $\text{NF-}\kappa\text{B}/\text{I}\kappa\text{B}\alpha$ complexes (Schmid et al., 2000). Over the 24 h of ponasterone A treatment, $\text{NF-}\kappa\text{B}/\text{I}\kappa\text{B}\alpha\text{M}$ complexes become prevalent because $\text{I}\kappa\text{B}\alpha\text{M}$ is not sensitive to $\text{I}\kappa\text{B}$ kinases and subsequent proteasome degradation (DiDonato et al., 1996). $\text{I}\kappa\text{B}\alpha$ that is not bound to $\text{NF-}\kappa\text{B}$, as a result of increasing competition with $\text{I}\kappa\text{B}\alpha\text{M}$, is degraded (Henkel et al., 1993) and therefore is not present at levels sufficient for detection by Western blot analysis.

$\text{I}\kappa\text{B}\alpha\text{M}$ Prevents Stress-Induced Activation of $\text{NF-}\kappa\text{B}$.

To determine whether the expressed $\text{I}\kappa\text{B}\alpha\text{M}$ was functionally active, we tested whether $\text{I}\kappa\text{B}\alpha\text{M}$ expression could inhibit $\text{NF-}\kappa\text{B}$ activation. Cells were exposed to BFA, hypoxia, or OA stress, and nuclear extracts were prepared at times shown previously to correspond with maximal stress-induced $\text{NF-}\kappa\text{B}$ activation (hypoxia for 2 h, 60 nM okadaic acid for 8 h, or 10 $\mu\text{g}/\text{ml}$ BFA for 2 h, followed by 2 h in BFA-free media) (Lin et al., 1998) and assayed for the presence of free $\text{NF-}\kappa\text{B}$ by EMSA. Our results show that BFA, OA, and hypoxia all induce $\text{NF-}\kappa\text{B}$ activation in both VCT cells treated with ponasterone A and noninduced $\text{I}\kappa\text{B}\alpha\text{M}$ cells (Figs. 3A and 4A). In our results, two bands of specific binding are detectable, which others have suggested are the p65/p50 (upper band) and p50/p50 forms (lower band) of $\text{NF-}\kappa\text{B}$ (Conant et al., 1994). $\text{I}\kappa\text{B}\alpha\text{M}$ cells pretreated with ponasterone A, however, had greatly reduced levels of BFA-, OA-, and hypoxia-induced $\text{NF-}\kappa\text{B}$ activation (Figs. 3A and 4A). To demonstrate the specificity of DNA binding, we performed competition experiments with nonlabeled $\text{NF-}\kappa\text{B}$ or AP-1 oligonucleotides. Figures 3B and 4B show that the addition of a 50-fold excess of $\text{NF-}\kappa\text{B}$ oligonucleotide effectively blocks the specific

interactions of $\text{NF-}\kappa\text{B}$ with the radiolabeled probe. The AP-1 oligonucleotide is the same length as the $\text{NF-}\kappa\text{B}$ oligonucleotide, but it is otherwise not related in sequence identity. The addition of a 50-fold excess of AP-1 oligonucleotide resulted in no change in binding of $\text{NF-}\kappa\text{B}$ to the labeled probe (Figs. 3B and 4B). Taken together, these results show that BFA, OA, and hypoxia activate $\text{NF-}\kappa\text{B}$ and that $\text{I}\kappa\text{B}\alpha\text{M}$ expression prevents stress-induced formation of free $\text{NF-}\kappa\text{B}$ in the nucleus.

To test whether $\text{I}\kappa\text{B}\alpha\text{M}$ expression inhibits $\text{NF-}\kappa\text{B}$ function, we transiently transfected cells with an $\text{NF-}\kappa\text{B}$ -sensitive luciferase reporter plasmid, $\text{pTk-}(\kappa\text{B})_6\text{-Luc}$. This plasmid contains a luciferase reporter gene downstream of a thymidine kinase promoter with six κB binding sites for $\text{NF-}\kappa\text{B}$ (Bachelier et al., 1991). After transfection, cells were treated with ponasterone A to induce $\text{I}\kappa\text{B}\alpha\text{M}$ expression and then were stress-treated (in the presence of ponasterone A) with

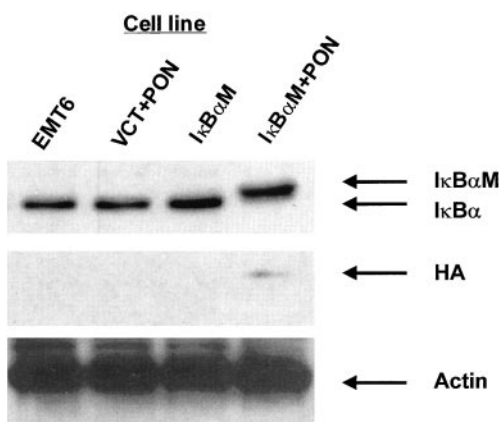


Fig. 2. Western blot analysis of inducible $\text{I}\kappa\text{B}\alpha\text{M}$ expression. EMT6 cells (EMT6) were transfected with either pIND and pVgRXR (VCT) or pVgRXR and pIND- $\text{I}\kappa\text{B}\alpha\text{M}$ ($\text{I}\kappa\text{B}\alpha\text{M}$). The $\text{I}\kappa\text{B}\alpha\text{M}$ also contains three HA tags, which increase the molecular weight of $\text{I}\kappa\text{B}\alpha\text{M}$ relative to wild-type $\text{I}\kappa\text{B}\alpha$. After treatment with 10 μM ponasterone A (PON) for 24 h, whole-cell lysates were collected and analyzed by Western blot. The resulting membranes were probed with anti- $\text{I}\kappa\text{B}\alpha$ (top), anti-HA (middle), and anti-actin (bottom) primary antibodies. Shown is a representative blot from one of three independent experiments.

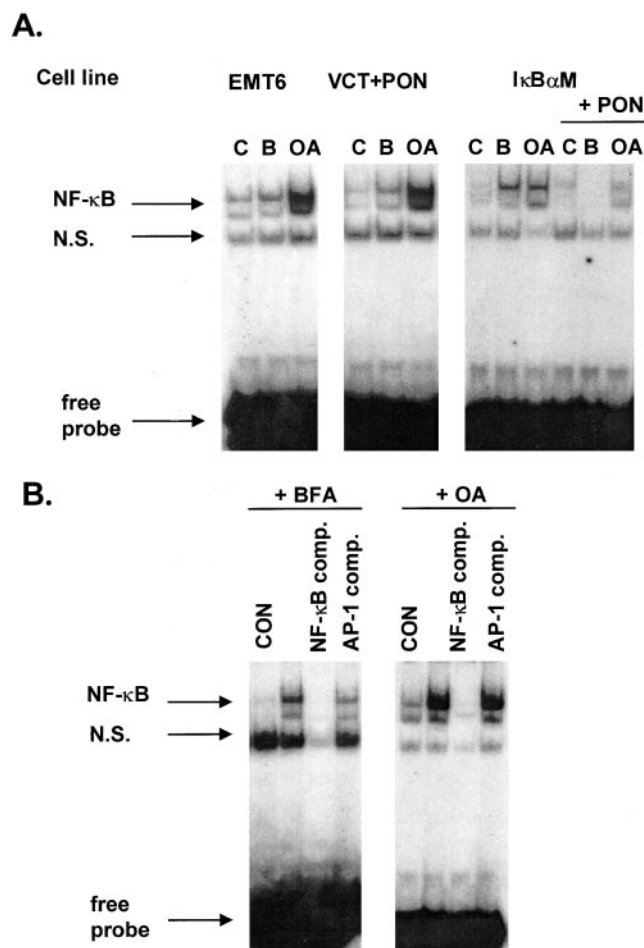


Fig. 3. A, $\text{I}\kappa\text{B}\alpha\text{M}$ expression prevents chemical stress-induced activation of $\text{NF-}\kappa\text{B}$. VCT and $\text{I}\kappa\text{B}\alpha\text{M}$ cells were treated with 10 μM ponasterone A (PON) for a total of 24 h. VCT, $\text{I}\kappa\text{B}\alpha\text{M}$, and nontransfected EMT6 cells (EMT6) were stress-treated in the presence of PON with 10 $\mu\text{g}/\text{ml}$ brefeldin A (B or BFA) for 2 h, followed by a BFA-free recovery for 2 h or 60 nM okadaic acid (OA) for 8 h (stress treatments previously shown to cause maximal $\text{NF-}\kappa\text{B}$ activation). After drug treatments, nuclear extracts from stress-treated and nonstressed (C) cells were harvested and analyzed by EMSA using a ^{32}P -labeled $\text{NF-}\kappa\text{B}$ oligonucleotide. Specific binding of $\text{NF-}\kappa\text{B}$ to the probe (NF- κB), nonspecific binding (N.S.), and unbound probe (free probe) bands are indicated. B, specificity of $\text{NF-}\kappa\text{B}$ binding by competition assay. Nuclear extracts from nonstressed (CON), BFA-, and OA-treated cells were incubated with oligonucleotide in the presence of a 50-fold excess of either unlabeled $\text{NF-}\kappa\text{B}$ or AP-1 oligonucleotide (NF- κB comp. and AP-1 comp., respectively).

either hypoxia for 2 h, 60 nM OA for 8 h, or 10 μ g/ml BFA for 2 h, followed by a 2 h recovery in BFA-free media (time points shown previously to correspond with maximal stress-induced NF- κ B activation). Cells were lysed, collected, and analyzed for luciferase expression by determining the light emission per sample in the presence of luciferol substrate. The relative luciferase activity obtained is indicative of the relative amount of functional NF- κ B for a given drug treatment. Figure 5 shows that treatment of EMT6 cells with BFA, hypoxia, or OA results in a marked increase in luciferase activity compared with activity observed in nonstressed cells (Fig. 5, A and B). VCT cells treated with ponasterone A and noninduced I κ B α M cells had similar increases in luciferase activity with stress treatment (Fig. 5, A and B). In contrast, I κ B α M cells treated with ponasterone A had significantly less BFA-, hypoxia-, and OA-induced luciferase activity (Fig. 5, A and B). These results suggest that I κ B α M expression blocks the formation of stress-induced free nuclear NF- κ B

and prevents enhanced NF- κ B *trans*-activation caused by stress treatment.

I κ B α M Expression Does Not Alter Etoposide Cytotoxicity in the Absence of Stress. Recent studies have reported that inhibition of NF- κ B activation enhances the toxicity of anticancer agents (Wang et al., 1996; Batra et al., 1999; Cusack et al., 2000; Huang et al., 2000). To determine the effects of I κ B α M expression on etoposide cytotoxicity in the absence of stress, VCT and I κ B α M cells were induced with ponasterone A for 24 h and then treated with etoposide for 1 h before analysis by colony-forming assay. Figure 6 shows that VCT cells treated with ponasterone A, noninduced I κ B α M cells, and I κ B α M cells treated with ponasterone A did not have significant changes in cell survival in the

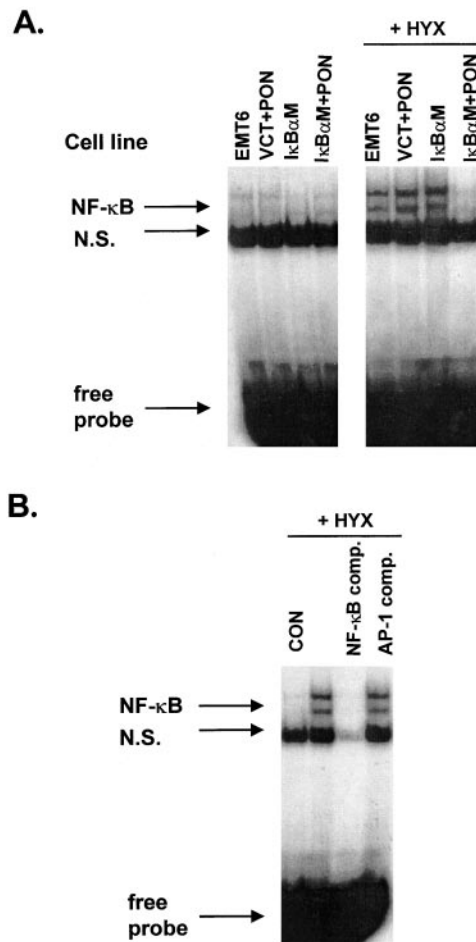


Fig. 4. A, I κ B α M expression prevents hypoxia-induced activation of NF- κ B. VCT and I κ B α M cells were treated with 10 μ M ponasterone A (PON) for 24 h. VCT, I κ B α M, and nontransfected EMT6 cells (EMT6) were then stress-treated with hypoxia (HYX) for 2 h (a stress treatment previously shown to cause maximal NF- κ B activation). After drug treatments, nuclear extracts were harvested and analyzed by EMSA using a 32 P-labeled NF- κ B oligonucleotide. Specific binding of NF- κ B to the probe (NF- κ B), nonspecific binding (N.S.), and unbound probe (free probe) bands are indicated. B, specificity of NF- κ B binding by competition assay. Nuclear extracts from nonstressed (CON) and hypoxia-treated cells were incubated with oligonucleotide in the presence of a 50-fold excess of either unlabeled NF- κ B or AP-1 oligonucleotide (NF- κ B comp. and AP-1 comp., respectively).

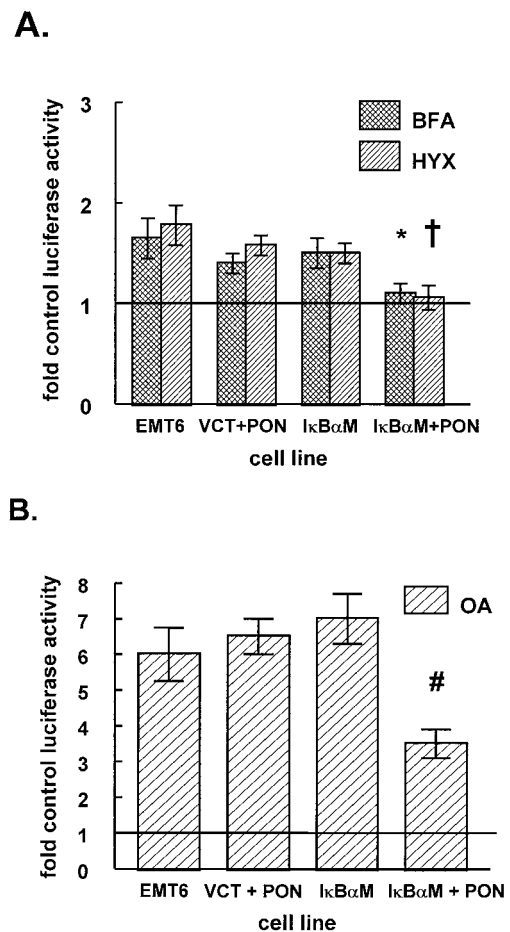


Fig. 5. I κ B α M expression inhibits stress-induced NF- κ B transactivation. EMT6, VCT, and I κ B α M cells were transiently transfected with a NF- κ B-sensitive luciferase reporter gene. After transfection, cells were treated with 10 μ M ponasterone A (PON) for a total of 24 h to induce gene expression. Cells were stress-treated in the presence of M ponasterone A with either 10 μ g/ml brefeldin A (BFA) for 2 h followed by a recovery in BFA-free media for 2 h, hypoxia (HYX) for 2 h (A), or 60 nM okadaic acid (OA) for 8 h (B) (time points previously shown to cause maximal NF- κ B activation). Results shown are the average fold-control luciferase activities from three to five independent experiments. Bars, S.E.M. *, a statistically significant decrease in luciferase activity was observed in BFA-treated I κ B α M cells induced with PON compared with noninduced BFA-treated I κ B α M cells. †, a statistically significant decrease in luciferase activity was observed in hypoxia-treated I κ B α M cells induced with PON compared with noninduced HYX-treated I κ B α M cells. #, a statistically significant decrease in luciferase activity was observed in OA-treated I κ B α M cells induced with PON compared with noninduced OA-treated I κ B α M cells (p < 0.05, ANOVA).

presence of etoposide compared with nontransfected EMT6 cells.

I κ B α M Expression Prevents BFA-, Hypoxia-, and OA-Induced Resistance to Etoposide. Our preliminary data with the proteasome inhibitor MG-132 suggested that inhibition of NF- κ B activation could reverse stress-induced resistance (Lin et al., 1998). To determine whether NF- κ B activation mediates stress-induced drug resistance, we assessed whether I κ B α M expression could prevent BFA-, hypoxia-, and OA-induced resistance to etoposide. I κ B α M cells were treated first with ponasterone A for 18 h and then with a stress treatment of hypoxia for 8 h, 60 nM OA for 8 h, or 10 μ M BFA for 2 h, followed by a recovery for 6 h in BFA-free media (in the continued presence of ponasterone A). During the last hour of stress, cells were treated with etoposide before analysis by colony-forming assay. Figure 7 shows that noninduced I κ B α M cells treated with BFA (Fig. 7A), hypoxia (Fig. 7B), or OA (Fig. 7C) are resistant to the cytotoxic effects of etoposide. Etoposide cytotoxicity in induced VCT cells treated with stress was not statistically different from that observed in noninduced I κ B α M cells treated with stress (data not shown). However, ponasterone-induced I κ B α M cells treated with BFA, hypoxia, or OA (Fig. 7, \square) were significantly more sensitive to the cytotoxic effects of etoposide compared with noninduced I κ B α M cells (Fig. 7, \blacksquare). I κ B α M expression partially but significantly reversed BFA-induced resistance to etoposide, whereas the reversal of hypoxia- and OA-induced drug resistance was almost complete. At nearly all doses of etoposide, the cell survival of induced I κ B α M cells treated with hypoxia or OA was not significantly different from the cell survival of nonstressed I κ B α M cells treated with etoposide alone. These data indicate that specific inhibition of NF- κ B attenuates both chemical- and physiological-induced resistance to etoposide.

Discussion

Most solid tumors are resistant to chemotherapy. This drug resistance has been attributed, in part, to the unique physiology of solid tumors. Oxygen deficiency (hypoxia), glucose deprivation, and acidosis are widespread conditions in

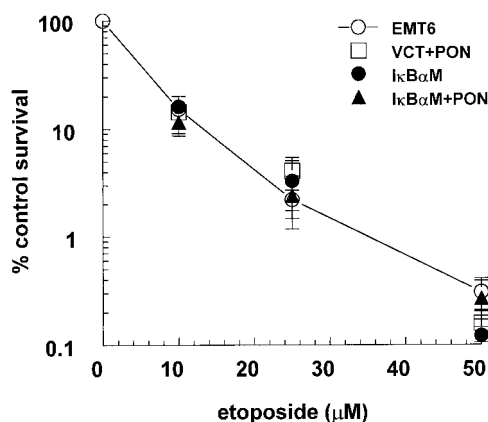


Fig. 6. I κ B α M expression and stable transfection do not alter etoposide cytotoxicity in the absence of stress. VCT, I κ B α M, and nontransfected EMT6 cells were treated with 10 μ M ponasterone A (PON) for a total of 24 h. Etoposide (10, 25, or 50 μ M) was added during the final hour of PON treatment before analysis by colony-forming assay. Results shown are the percentage of control cell survival averages of triplicate plates from at least three independent experiments. bars, S.E.M.

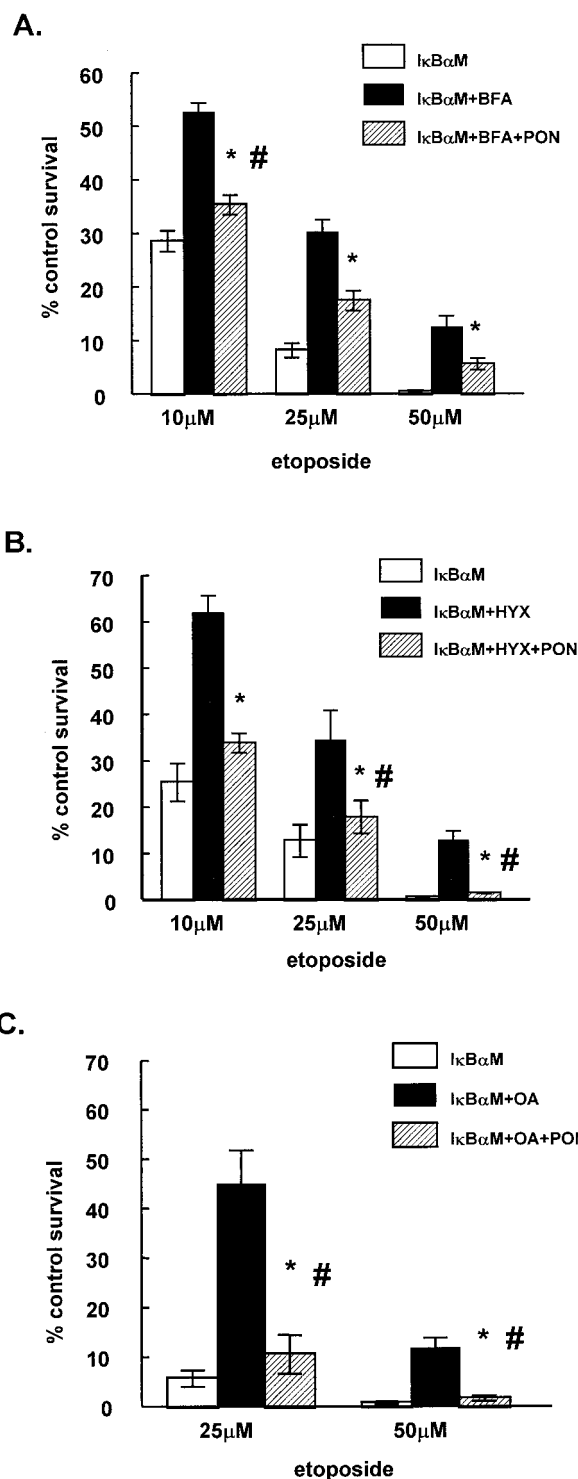


Fig. 7. I κ B α M expression prevents BFA-, HYX- and OA-induced resistance to etoposide. I κ B α M cells were treated with 10 μ M ponasterone A (PON) for a total of 24 h. Cells were stress-treated in the presence of PON with 10 μ M brefeldin A (BFA) for 2 h, followed by a recovery in BFA-free media for 6 h (A), hypoxia for 8 h (B), or 60 nM okadaic acid (OA) for 8 h (C). Etoposide (10, 25, or 50 μ M) was added during the final hour of PON and stress treatment before analysis by clonogenicity assay. Results shown are the percentage of control cell survival averages of triplicate plates from at least three independent experiments. Bars, S.E.M. *, a statistically significant decrease in survival of induced I κ B α M cells treated with stress was observed compared with noninduced I κ B α M cells treated with stress. #, there was no statistically significant change in survival of induced I κ B α M cells treated with stress compared with non-stressed I κ B α M cells ($p < 0.05$, ANOVA).

solid tumors. Hypoxia has been shown repeatedly to limit the responsiveness of tumor cells to ionizing radiation (Bush et al., 1978) and chemotherapeutic agents (Sakata et al., 1991). Mechanisms by which hypoxic cells develop resistance to radiation and chemotherapy may involve low oxygen tension and poor drug penetration into solid tumors (Durand, 1989). Hypoxia treatment is also known to cause gene amplification, cell-cycle arrest, and altered cell-cycle distribution (Stoler et al., 1992; Amellem and Pettersen, 1997). Alterations in gene expression and cell-cycle progression mediated by hypoxia-activated proteins, such as the hypoxia-inducible factor, HIF-1 α (Carmeliet et al., 1998), may be involved in the resistance of tumor cells to cancer chemotherapeutic drugs.

Another type of resistance that develops in cells exposed to hypoxia may be associated with the induction of specific stress-responsive proteins and transcription factors. At the cellular level, the ER responds to stress by three distinct signaling mechanisms. One pathway, the unfolded protein response (UPR), is activated by the presence of abnormally folded proteins in the ER and results in production of the glucose-regulated protein GRP78 (Pahl, 1999). The EOR results in activation of the nuclear transcription factor NF- κ B by the accumulation of normally folded proteins in the ER (Pahl, 1999). The third, the sterol regulatory cascade, is induced by the depletion of cholesterol (Pahl, 1999).

Evidence now suggests that the activation of ER stress pathways may explain the intrinsic insensitivity of solid tumors to chemotherapy. Stress conditions associated with solid tumors, such as hypoxia, induce the expression of glucose-regulated proteins (Wilson et al., 1989), heat-shock proteins (Patel et al., 1995), stress-activated protein kinases (Conrad et al., 2000), and NF- κ B (Koong et al., 1998) and resistance to anticancer agents (Wilson et al., 1989). Analysis of human breast tumors has determined that GRP78 levels are elevated in malignant but not in nonmalignant lesions (Fernandez et al., 2000). The activation of ER stress responses has been further correlated with the development of resistance to anticancer agents that inhibit topoisomerase II. Treatment with the glucose-regulated stresses 2-deoxyglucose, glucosamine, calcium ionophore, or tunicamycin results in activation of NF- κ B (Pahl and Baeuerle, 1997; Pahl, 1999) and the development of resistance to teniposide, etoposide, and doxorubicin (Adriamycin) (Hughes et al., 1989; Lin et al., 1998). In the present study, we show that EMT6 cells treated with other chemical stress agents, BFA or OA, or the physiological stress agent hypoxia, result in similar levels of resistance to etoposide. This finding implies that the mechanism of stress-induced resistance to etoposide may be through the activation of ER stress responses.

Our previous work suggests that the EOR pathway mediates stress-induced resistance to etoposide. We have shown that BFA treatment induces both the UPR and EOR stress pathways and causes the development of resistance to the topoisomerase II inhibitor teniposide (Lin et al., 1998). This study showed that selective activation of the EOR pathway with OA also results in the development of resistance to teniposide to an extent similar to that observed with BFA treatment (Lin et al., 1998). Selective activation of the UPR pathway with the glucosidase inhibitor castanospermine resulted in no change in sensitivity to teniposide even though it markedly increased GRP78 levels (Lin et al., 1998). Furthermore, inhibition of NF- κ B activation with MG-132 or prosta-

glandin A₁ is sufficient to reverse BFA-induced resistance to teniposide (Lin et al., 1998; Y. C. Boller, et al., manuscript in preparation). Taken together, these data suggested the hypothesis that activation of the EOR pathway through the release of NF- κ B is the mechanism by which EMT6 cells develop resistance to etoposide.

To study the role of the EOR pathway in stress-induced drug resistance, a phosphorylation site-deficient mutant of I κ B α (I κ B α M) was used to selectively inhibit NF- κ B activation. Inducible expression of I κ B α M resulted in virtually no detectable wild-type I κ B α . When introduced into cells, I κ B α M probably becomes the major NF- κ B/I κ B complex because of the high on-off rate of NF- κ B/I κ B α binding kinetics (Schmid et al., 2000). Over time, NF- κ B/I κ B α M complexes become predominant over NF- κ B/I κ B α complexes because I κ B α M cannot be phosphorylated by I κ B kinases and degraded (DiDonato et al., 1996). Free I κ B α that has dissociated from NF- κ B is degraded by proteasomes and thus does not appear in protein collections from I κ B α M cells treated with ponasterone A for 24 h (Henkel et al., 1996).

In this study, we show that expression of I κ B α M suppresses stress-induced NF- κ B activation. Previous time-course studies in our laboratory have shown that maximal stress-induced NF- κ B activation occurs 2 h after hypoxia treatment, 4 h after BFA treatment, and 8 h after OA treatment (Lin et al., 1998; data not shown). These time points were used in the present study to ascertain whether I κ B α M expression could inhibit the maximal NF- κ B response to stress. In both EMSA and luciferase reporter gene assays, VCT cells treated with ponasterone A and noninduced I κ B α M cells display enhanced NF- κ B activation with stress. Ponasterone A treatment alone does not activate NF- κ B or interfere with stress-induced NF- κ B activation in EMT6 cells (data not shown). Despite the differences in NF- κ B activation kinetics, I κ B α M cells treated with ponasterone A were virtually insensitive to stress-induced NF- κ B activation. The expression of I κ B α M also greatly inhibited NF- κ B activation induced by OA, a relatively stronger activator of NF- κ B (Lin et al., 1998). These data show that the inducible I κ B α M was useful for testing the effects of selective inhibition of NF- κ B on drug resistance.

The effects of I κ B α M expression on etoposide toxicity were determined. Others have observed that I κ B α M expression enhances the toxicity of anticancer agents such as camptothecin, paclitaxel, daunorubicin, and tumor necrosis factor (Wang et al., 1996; Batra et al., 1999; Cusack et al., 2000; Huang et al., 2000). However, we observed no significant change in cell survival of induced I κ B α M cells treated with etoposide compared with noninduced I κ B α M cells. These data suggest that NF- κ B activation does not influence the cytotoxicity of topoisomerase II inhibitors in the absence of stress in our murine cell line.

We have demonstrated that inducible I κ B α M expression prevents drug resistance caused by BFA, hypoxia, and OA. Noninduced I κ B α M cells exhibit levels of BFA-, hypoxia-, and OA-induced resistance to etoposide similar to those levels observed in wild-type EMT6 cells. Induction of I κ B α M, in contrast, results in significantly greater drug toxicity in the presence of stress. The abrogation of drug resistance was essentially complete, because hypoxia- or OA-treated I κ B α M cells induced with ponasterone A had etoposide toxicity levels

that were not significantly different from those of non-stressed cells.

Taken together, our data clearly show that NF- κ B activation plays a critical role in both chemical and physiological resistance to etoposide. Although statistically significant, the reversal of stress-induced drug resistance with I κ B α M expression was not complete with the stress agent BFA. However, reversal of hypoxia- or OA-induced resistance was complete. Although there is evidence that I κ B kinases, which play a primary role in I κ B phosphorylation, may activate additional signaling pathways (Hu et al., 2001), evidence for the direct effects of I κ B α on other signaling pathways is not available. Other pathways independent of NF- κ B activation may contribute to stress-induced drug resistance, but the data presented here show that NF- κ B activation plays a major role in stress-induced drug resistance. Our data further imply that relatively small changes in NF- κ B activation can have dramatic effects on cell viability, suggesting that inhibition of NF- κ B activation may result in the modulation of pleiotropic responses with biological and therapeutic significance. The concentrations of etoposide used in our studies are within the range of plasma concentrations of etoposide that are obtained clinically (Chen and Uckun, 2000). Therefore, it is likely that stress-induced resistance to topoisomerase II inhibitors play a role in the intrinsic resistance of solid tumors to topoisomerase II-directed agents. These findings also suggest that coadministration of agents that inhibit the activation of NF- κ B would enhance the efficacy of topoisomerase II inhibitors in the treatment of cancer. Inhibition of NF- κ B activation with I κ B α M is known to enhance the toxicity of many anticancer agents both in vivo and in vitro (Wang et al., 1996; Batra et al., 1999; Cusack et al., 2000; Huang et al., 2000). Agents that inhibit NF- κ B activation, such as the proteasome inhibitors PS-341 and lactacystin, enhance chemotherapeutic efficacy in in vivo tumor assays (Teicher et al., 1999; Ogiso et al., 2000). These studies and those reported here suggest that the interruption of signaling pathways mediating intrinsic drug resistance, such as physiological stress, represents new therapeutic targets for cancer drug therapy.

Acknowledgments

We thank Dr. Michael Karin of the University of California, San Diego, for providing the I κ B α M (S32/36A) construct and Dr. Heike Pahl of the University Hospital, Freiburg, Germany, for providing the NF- κ B-sensitive luciferase reporter construct.

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