Nicotine Inactivation of the Proapoptotic Function of Bax through Phosphorylation*

Nicotine-induced cell survival is associated with chemoresistance of human lung cancer cells, but our understanding of the intracellular mechanism(s) is fragmentary. Bax is a major proapoptotic member of the Bcl2 family and a molecule required for apoptotic cell death. Growth factor (i.e. granulocyte-macrophage colony-stimulating factor)-induced phosphorylation of Bax has been reported to negatively regulate its proapoptotic function. Because Bax is ubiquitously expressed in both small cell lung cancer and non-small cell lung cancer cells, nicotine may mimic growth factor(s) to regulate the activity of Bax. We found that nicotine potently induces Bax phosphorylation at Ser-184, which results in abrogation of the proapoptotic activity of Bax and increased cell survival. AKT, a known physiological Bax kinase, is activated by nicotine, co-localizes with Bax in the cytoplasm, and can directly phosphorylate Bax in vitro. Treatment of cells with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 or specific depletion of AKT expression by RNA interference can block both nicotine-induced Bax phosphorylation and cell survival. Importantly, nicotine-induced Bax phosphorylation potently blocks stress-induced translocation of Bax from cytosol to mitochondria, impairs Bax insertion into mitochondrial membranes, and reduces the half-life of Bax protein (i.e. from 9–12 h to <6 h). Because knockdown of Bax expression by gene silencing results in prolonged cell survival following treatment with cisplatin in the absence or presence of nicotine, Bax may be an essential component in the nicotine survival signaling pathway. Thus, nicotine-induced survival and chemoresistance of human lung cancer cells may occur in a novel mechanism involving activation of PI3K/AKT that directly phosphorylates and inactivates the proapoptotic function of Bax.

Lung cancer is the leading cause of cancer-related mortality and kills more people than cancers of the breast, prostate, colon, and pancreas combined in the United States (1). More than 90% of lung cancer is related to cigarette smoke, which contains about 4,000 chemicals, 55 of which have been evaluated as carcinogens (2–4). Nicotine, a major component in tobacco, exists at high concentrations (90–1000 nm) in the blood of smokers (2). High affinity nicotine acetylcholine receptors (nAChRs) are found on both human lung cancer and normal lung cells (5, 6). Nicotine not only promotes lung cancer development by activating the cell growth pathways but also reduces the efficacy of chemotherapeutic agents by stimulating survival pathways (7–9). However, the intracellular mechanism(s) by which nicotine induces survival and chemoresistance of human lung cancer cells remains elusive.

Cell death through apoptosis may be a potential mechanism for lung cancer treatment. Apoptosis occurs by activation of an intrinsic or extrinsic pathway and can be divided into three interdependent phases: induction, decision, and execution. The decision phase is largely regulated by the Bcl-2 family of apoptotic regulators, which comprises three subfamilies (10–11). The subfamily that includes Bcl2 and Bel-XL members blocks apoptosis, whereas the Bax subfamily, consisting of Bax and Bak, and the BH3-only subfamily, including Bad, Bid, Bok, Bik, and Bim, promote apoptosis (12–17). Bcl2 family members function in a tightly regulated network that protects the mitochondria or induces mitochondrial dysfunction. Studies have determined the functional significance of the conserved Bcl2 homology domains (BH1, BH2, BH3, and BH4) as well as the hydrophobic transmembrane domain that localizes the members of this family to the outer mitochondrial membrane. It is popularly held that antiapoptotic Bcl2 and Bel-XL heterodimerize with proapoptotic Bax or Bak such that the hydrophobic crevices on their surfaces bind to the exposed BH3 domain of Bax or Bak to block their proapoptotic function. Thus, heterodimerization appears to regulate, at least in part, cell survival or death (14). Further, the BH3 domain of the proapoptotic members is required for both their oligomerization and killing activity, although homodimerization does not necessarily correlate with killing activity (18). Genetic studies using Bax and Bak single and double homozygous knock-out mice reveal that either Bax or Bak is essential for inducing mitochondrial dysfunction characterized by the release of potent caspase activators, including cytochrome c and Smac/Dia-blo, which initiate the intrinsic cell death pathway (19).

Bax, the major promoter of cell death, has been identified as a promising prognostic indicator in patients with lung cancer (20). However, the mechanism(s) involved in regulating the proapoptotic activity of Bax is not fully understood. It has been proposed that activation of the proapoptotic function of Bax likely occurs through several interdependent mechanisms, including translocation from cytosol to mitochondria (21), oligomerization, and insertion into mitochondrial membranes fol-

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allowing stress (22–24). In healthy cells, Bax is predominantly a soluble monomeric protein, despite the fact that it possesses a C-terminal hydrophobic segment (25). This hydrophobic domain, unlike those of Bcl2 and Bcl-XL, is sequestered inside a hydrophobic cleft (12, 22). Bax is translocated into mitochondria upon induction of apoptosis (26–27). This translocation process appears to involve a conformational change in Bax to expose its C-terminal hydrophobic domain (28). After translocation, Bax forms large oligomers, which insert into mitochondrial membranes, leading to cytochrome c release and cytotoxic activities. However, this has not been seen in other Bcl2 family proteins, such as Bid and Bad (23).

A recent report indicated that GM-CSF induces Bax phosphorylation at Ser-184 in the hydrophobic C-terminal tail and activates the proapoptotic activity of Bax in neutrophils (29). These findings reveal that the proapoptotic activity of Bax can be regulated by a post-translational modification (i.e. phosphorylation). Because inactivation of Bax has been found to promote tumorigenesis by rendering the tumor cells less susceptible to cell death (30), nicotine may promote lung cancer development in a novel mechanism involving regulation of the proapoptotic activity of Bax. In this study, we demonstrated that nicotine can induce activation of PI3K/AKT, which directly phosphorylates and inactivates Bax in human lung cancer cells.

### EXPERIMENTAL PROCEDURES

**Materials**—Nicotine, propanolol, etosid (VP-16), and cisplatin were purchased from Sigma. LY294002 was obtained from Calbiochem. AKT and phospho-specific AKT antibodies were purchased from Cell Signaling Technology (Beverly, MA). AKT siRNA, anti-Bax, fluorescein isothiocyanate-conjugated anti-mouse IgG, and rhodamine-conjugated anti-rabbit IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). GFP-WT, GFP-S184A, and GFP-S184E Bax cDNAs were kindly provided by Dr. Richard J. Youle (National Institutes of Health, Bethesda, MD). [32P]Orthophosphate and [γ-32P]ATP were purchased from MP Biomedicals (Irvine, CA). Purified active AKT was obtained from Upstate Biotechnology (Lake Placid, NY). pSilencerTM 2.1-U6 hygromycin vector was purchased from Ambion (Austin, TX). All reagents used were obtained from commercial sources unless otherwise stated.

**Cell Lines, Plasmids, and Transfections—**A549 cells were maintained in F-12K medium with 10% fetal bovine serum and 4 mM l-glutamine. NCI-H69, NCI-H82, NCI-H1157, and NCI-H460 cells were maintained in RPMI 1640 with 10% fetal bovine serum. The pcDNA3 plasmids bearing GFP-WT, GFP-S184A, or GFP-S184E Bax cDNA were transfected into H1157 cells using Lipofectamine™ 2000 according to the manufacturer's instructions. The endogenous Bax was immunoprecipitated using an agarose-conjugated Bax antibody, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. The membrane slice containing Bax was excised and hydrolyzed in 5.7 M HCl (Pierce) at 110 °C for 90 min under a vacuum. Cold phosphoserine, phosphothreonine, and phosphotyrosine were added to the sample; they were applied together onto a TLC plate and separated by electrophoresis utilizing a Multiphor II apparatus (Amersham Biosciences) with an equal volume of pH 1.9 and pH 3.5 buffers as described previously (24). The location of phosphoamino acids was determined by ninhydrin staining and autoradiography.

**Phosphorylation of Bax in Vitro—**Purified recombinant Bax protein was obtained from Protein X, Inc. (San Diego, CA). The endogenous Bax was immunoprecipitated using an agarose-conjugated Bax antibody from lysates of A549 cells. Recombinant or endogenous Bax was resuspended in a kinase assay buffer containing 1 μCi of [γ-32P]ATP, 10 μM ATP, 75 mM MgCl₂, 1 mM dithiothreitol, 1 μM NaF, and Tris buffer pH 7.5, and incubated with purified active AKT at 30 °C for various times as indicated. The reaction was terminated by the addition of SDS sample buffer and boiling prior to SDS-PAGE. Phosphorylation of Bax was determined by autoradiography.

**Immunofluorescence—**A549 cells were grown on a poly-lysine-coated cover slide, washed with 1× PBS, fixed with ice-cold methanol, and blocked with 10% donkey serum. Then, cells were incubated with mouse against human Bax and rabbit against human AKT primary antibodies for 90 min. After washing, samples were unbroken with fluorescein isothiocyanate-conjugated anti-mouse and rhodamine-conjugated anti-rabbit secondary antibodies for 60 min. Cells were washed with 1× PBS and observed under a fluorescent microscope (Zeiss). Pictures were taken and colored with the same exposure setting for each experiment. To determine subcellular regions of protein co-localization, individual red- and green-stained images derived from the same field were merged using the Adobe Photoshop software from Adobe Systems Inc. (Lexington, MA). Areas of protein co-localization appear in yellow (see Fig. 3).

**Subcellular Fractionation—**Cells (2×10⁶) were washed with cold 1× PBS and resuspended in isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM Hepes, pH 7.5) containing protease inhibitor mixture set I, homogenized with a polytron homogenizer operating for four bursts of 10 s each at a setting of 5, and then centrifuged for 2000× g for 3 min to pellet the nucleus. The supernatant was centrifuged at 13,000× g for 10 min to pellet mitochondria as described previously (31). The second supernatant was further centrifuged at 150,000× g for 30 min to pellet light membranes. The resulting supernatant is the cytosolic fraction. Mitochondria were washed twice with mitochondrial buffer and resuspended in 1% Nonidet P-40 lysis buffer, rocked for 60 min, and then centrifuged at 17,530× g for 10 min at 4 °C. The supernatant containing the nonextractable mitochondrial membrane proteins was collected. Protein (100 μg) from each fraction was subjected to SDS-PAGE. Bax was analyzed by Western blot using a Bax antibody. The purity of fractions was confirmed by assessing localization of the mitochondria-specific protein prohibitin (33).

**Alkali Extraction of Bax Peripherally Associated with Mitochondrial Membrane—**Cells were treated with cisplatin (40 μM) in the absence or presence of nicotine (1 μM) for various times. Mitochondria were isolated by subcellular fractionation, resuspended in freshly prepared 0.1 M Na₂CO₃, pH 11, and incubated on ice for 30 min. The samples were then centrifuged at 200,000× g for 30 min. The alkali-extracted membrane pellet was resuspended with 1% Nonidet P-40 lysis buffer, rocked for 60 min, and then centrifuged at 17,530× g for 10 min at 4 °C. The supernatant containing the nonextractable membrane proteins was collected and subjected to SDS-PAGE. The alkali-resistant Bax (i.e. nonextractable or integral) was determined by Western blot using a Bax antibody as described previously (21, 34).

**Depletion of AKT Expression by RNA Interference (RNAi)—**Human AKT siRNA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A549 cells were transfected with AKT siRNA using Lipofectamine™ 2000 according to the manufacturer’s instructions. A control siRNA (non-homologous to any known gene sequence) was used as a negative control. The levels of AKT expression were analyzed by Western blotting using an AKT antibody. Bax phosphorylation or cell viability was assessed following various treatments. Specific silencing of the targeted AKT gene was confirmed by at least three independent experiments.

**Vector-based Gene Silencing of Bax by RNAi—**The Bax DNA target sequence for siRNA design is AACTGATCGAGAACCATTGG. This was determined by the siRNA Target Finder (Ambion) according to human Bax cDNA sequence. The Bax-specific hairpin siRNA insert (sense-loop-antisense) was designed using a computerized design tool based on target sequence following instructions from Ambion. The pcDNA3 plasmid bearing the nucleotide-encoding Bax-specific hairpin siRNA insert was synthesized and ligated into pSilencer™ 2.1-U6 hygromycin vector. The pSilencer™ 2.1-U6 hygromycin plasmids bearing Bax hairpin siRNAs were transfected into A549 cells using Lipofectamine™ 2000 according to the manufacturer’s instructions. The stable clones persistently producing Bax siRNA were selected using hygromycin (0.8 mg/ml). The levels of Bax expression were analyzed by Western blot using a Bax antibody.
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**RESULTS**

Cell Viability Assay—The apoptotic and viable cells were detected using an ApoAlert Annexin-V kit from Clontech according to the manufacturer's instructions. The percentage of viable cells or apoptotic cells was determined by fluorescence-activated cell sorter analysis as described previously (35). Cell viability was also confirmed using the trypan blue dye exclusion method (32).

**Nicotine Induces Bax Phosphorylation in Association with Increased Survival of Human Lung Cancer Cells**—A recent report shows that GM-CSF can induce Bax phosphorylation through activation of PI3K/AKT, which results in markedly decreased proapoptotic activity of Bax (29). Because nicotine can potently induce activation of PI3K/AKT (36), it is possible that nicotine may regulate the function of Bax in a mechanism involving its phosphorylation. To test this, A549 cells expressing high levels of endogenous Bax were metabolically labeled with $[^{32}P]$orthophosphoric acid and treated with nicotine (1 μM) for 60 min. Phosphorylation of Bax was analyzed by autoradiography. Results indicate that nicotine potently stimulates serine phosphorylation of Bax (Fig. 1, B and C). To test a potential role for nicotine-stimulated Bax phosphorylation, A549 cells were treated with the chemotherapeutic agent cisplatin, which is widely used in the treatment of patients with lung cancer, in the presence or absence of nicotine for various times. Cell viability indicates that nicotine significantly prolongs survival of A549 cells following cisplatin treatment (Fig. 1D). These findings suggest that nicotine-induced survival and chemoresistance may occur, at least in part, in a mechanism involving Bax phosphorylation.

Nicotine Induces Bax Phosphorylation at Ser-184, Which Results in Inactivation of the Proapoptotic Function of Bax—GM-CSF can induce Bax phosphorylation at Ser-184, which is located in the C-terminal hydrophobic domain (29). Phosphoamino acid analysis reveals that nicotine-induced Bax phosphorylation is located at serine amino acid(s) (Fig. 1C). To test whether nicotine induces Bax phosphorylation at Ser-184, WT, S184A, or S184E cDNA in the pcDNA3 mammalian expression vector was transfected into H157 cells using Lipofectamine™ 2000. We chose this cell line because H157 cells express relatively low levels of endogenous Bax (Fig. 1A). Thus, the proapoptotic function of individual Bax mutants could be more accurately evaluated because any possible effect from endogenous Bax could be minimized. After a 48-h transfection, cells were metabolically labeled with $[^{32}P]$orthophosphoric acid and treated with nicotine (1 μM), and phosphorylation of Bax was analyzed as described under “Experimental Procedures.” In the meantime, cells were treated with the chemotherapeutic drug cisplatin (40 μM) in the absence or presence of nicotine for 24 h. Cell viability was assessed using an ApoAlert Annexin-V kit. Results indicate that nicotine induces phosphorylation of WT but not the S184A or S184E mutant Bax (Fig. 2A), suggesting that nicotine stimulates Bax phosphorylation exclusively at the Ser-184 site. Importantly, expression of the nonphosphorylatable S184A results in more apoptotic cell death than WT. Nicotine can prolong the survival of cells expressing WT Bax but not the S184A Bax mutant (Fig. 2B). By contrast, the phosphomimetic S184E Bax exhibits no apoptotic activity. Nicotine has no additional survival effect in cells expressing S184E Bax mutant (Fig. 2B). These findings reveal that either nicotine-induced Ser-184 site phosphorylation or genetically mimicking Ser-184 site phosphorylation (i.e. S184E) results in abrogation of the proapoptotic function of Bax.

AKT Is Co-localized with Bax in Cytoplasm and Activated by Nicotine, and the Active AKT Directly Phosphorylates Bax in Vitro—To assess a potential direct role for AKT as a physiological Bax kinase, subcellular distribution of AKT and Bax was assessed by immunofluorescent staining. A mouse antibody against human Bax, a rabbit antibody against human AKT, and fluorescein isothiocyanate-conjugated anti-mouse (green) or rhodamine-conjugated anti-rabbit (red) secondary antibodies were used so that cells could be stained simultaneously without cross-reaction. Results indicate that Bax is primarily co-localized with AKT in the cytoplasm of A549 cells (Fig. 3A).

To test whether AKT is a nicotine-activated Bax kinase, nicotine was assessed for its ability to activate AKT in A549 cells. Cells were treated with increasing concentrations of nicotine for 60 min. Phosphorylation of AKT was analyzed by Western blot using a phospho-specific AKT antibody. Results indicated that nicotine can potently activate AKT in a dose-dependent manner (Fig. 3B). To determine whether AKT can directly phosphorylate endogenous Bax, Bax protein was immunopre-
Cisplatin was found to decrease cell viability in H157 cells, which express relatively low levels of endogenous Bax, using Lipofectamine™ 2000. After transfection, cells were metabolically labeled with [32P]orthophosphoric acid and treated with nicotine for 60 min. Phosphorylation of Bax was then analyzed as described in the legend for Fig. 1. B, the pcDNA3 plasmids bearing GFP-WT, GFP-S184A, or GFP-S184E were transfected into H157 cells, which express relatively low levels of endogenous Bax, using Lipofectamine™ 2000. After 48 h, cells were treated with cisplatin (Cis) in the absence or presence of nicotine for 24 h. Cell viability was then analyzed as described in the legend for Fig. 1D. Data represent the mean ± S.D. of three determinations.

The PI3K-specific Inhibitor LY294002 Blocks Nicotine-induced Bax Phosphorylation and Promotes Apoptosis—To pharmacologically demonstrate a functional role of AKT as a physiological nicotine-activated Bax kinase in vivo, LY294002, a PI3K-specific inhibitor that can block the PI3K/AKT signaling pathway (37), was used. A549 cells were metabolically labeled with [32P]orthophosphoric acid and treated with nicotine (1 μM) in the absence or presence of increasing concentrations of LY294002 (0.1–10 μM) for 60 min. Results indicate that LY294002 can block nicotine-induced Bax phosphorylation in a dose-dependent manner (Fig. 4). Functionally, LY294002 inactivates AKT in a kinase assay buffer containing [γ-32P]ATP as described under “Experimental Procedures.” Western blot analysis of AKT was performed to confirm and quantify Bax protein (Fig. 3D). These findings suggest that nicotine-activated AKT has the potential to directly phosphorylate and inactivate Bax in human lung cancer cells.

Depletion of AKT Expression by RNAi Blocks Both Nicotine-induced Bax Phosphorylation and Survival—Our findings strongly indicated that AKT functions as a nicotine-activated Bax kinase in human lung cancer cells (Figs. 3 and 4). To test whether AKT is required for nicotine-stimulated Bax phosphorylation, an RNAi approach was employed. It was recently demonstrated that 21-base double-stranded RNA (siRNA) is a potent mediator of the RNA interference effect in mammalian cells (38). A549 cells were transfected with AKT siRNA as described under “Experimental Procedures.” Results show that the AKT siRNA efficiently and specifically reduced AKT expression in A549 cells, whereas control siRNA had no effect (Fig. 5A). Importantly, specific disruption of AKT expression by RNAi blocked nicotine-induced Bax phosphorylation and significantly enhanced apoptotic cell death following treatment of cells with cisplatin (Fig. 5). These findings indicate that AKT is a required kinase for nicotine-induced Bax phosphorylation and survival of human lung cancer cells.

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The β-Adrenergic Receptor-specific Inhibitor Propranolol Potently Blocks Nicotine-induced Bax Phosphorylation and Enhances Apoptosis—nAChRs, especially α7nAChR or β-adrenergic receptor, have been found to play an important role in nicotine signaling in human lung cancer cells (31). Thus, α-bungarotoxin, a non-competent potent α7nAChR-specific inhibitor (39), and propranolol, a β-adrenergic receptor inhibitor (40), were selected to test which type of receptor may be involved in nicotine/Bax signaling. A549 cells were treated with nicotine in the presence or absence of increasing concentrations of propranolol or α-bungarotoxin. Interestingly, propranolol but not α-bungarotoxin can block nicotine-induced Bax phosphorylation in a dose-dependent manner (Fig. 6A and data not shown).
A549 cells were metabolically labeled with \(^{32}\text{P}\)orthophosphoric acid and treated with nicotine (1 \(\mu\text{M}\)) in the absence or presence of increasing concentrations of LY294002 for 60 min. Phosphorylation of Bax was analyzed as described in the legend for Fig. 1B. A549 cells were treated with cisplatin (Cis, 40 \(\mu\text{M}\)) in the absence or presence of nicotine (1 \(\mu\text{M}\)) or increasing concentrations of LY294002 for 48 h. Cell viability was assessed as described in the legend for Fig. 1D. Data represent the mean \pm S.D. of three determinations.

Phosphorylation of Bax at Ser-184 Results in Retention of Bax in Cytosol and Failure to Target Mitochondria—Our data show that nicotine can induce Bax phosphorylation at the Ser-184 site, and phosphorylation at this site resulted in inactivation of the proapoptotic function of Bax (Fig. 2). However, the intracellular mechanism(s) remains unclear. It is well known that the proapoptotic activity of Bax is dependent on its cytosolic or mitochondrial localization (21). To genetically test whether phosphorylation of Bax affects its subcellular localization, GFP-tagged WT, the nonphosphorylatable S184A, and the phosphomimetic S184E Bax mutants were transfected into H157 cells. Subcellular fractionation experiments were performed to isolate mitochondria and cytosol as described under “Experimental Procedures.” The exogenous WT or mutant Bax forms to isolate mitochondria and cytosol as described under "Experimental Procedures." The exogenous WT or mutant Bax antibody. Results indicate that the majority of WT Bax is in the cytosol, and only a small amount of WT Bax is localized on mitochondrial membranes. By contrast, the phosphomimetic S184E mutant Bax is observed only in the cytosol, whereas the nonphosphorylatable S184A Bax mutant is detected only in mitochondrial fraction (Fig. 7). These findings help to explain why the nonphosphorylatable S184A Bax mutant has more mitochondrial fraction (Fig. 7), this indicates that mitochondrial and cytosolic fractions are highly pure without cross-contamination.

Nicotine-induced Bax Phosphorylation Blocks Cisplatin-induced Bax Translocation to Mitochondria from Cytosol as well as Insertion into Mitochondrial Membranes and Reduces the Half-life of Bax Protein—Bax contains a single predicted transmembrane domain at its C terminus. Bax is located in the cytosol and is peripherally associated with mitochondrial membranes in unstimulated cells (21). Interestingly, Bax can be translocated and inserted into mitochondrial membranes following a death signal (21). To test whether nicotine-induced Bax phosphorylation affects stress-induced translocation of Bax, A549 cells were treated with cisplatin (40 \(\mu\text{M}\)) in the absence or presence of nicotine (1 \(\mu\text{M}\)) for 24 h. Mitochondria and cytosol were isolated, and Bax was analyzed using a Bax antibody. Results reveal that treatment of cells with cisplatin resulted in accumulation of Bax in mitochondrial membranes, and nicotine can potently inhibit cisplatin-induced Bax translocation to mitochondria from cytosol (Fig. 8A). It has been demonstrated that an alkali extraction experiment can distinguish whether Bax protein slightly associates with or inserts into mitochondrial membranes (21). An alkali extraction experiment was employed to test whether nicotine-induced Bax phosphorylation can affect Bax insertion into mitochondrial membranes following stress. A549 cells were treated with cisplatin in the absence or presence of nicotine for various times as indicated. Mitochondria were isolated and incubated in 0.1 M
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**Fig. 6.** The β-adrenergic receptor-specific inhibitor propranolol potently inhibits nicotine-induced Bax phosphorylation and enhances apoptosis. A, A549 cells were metabolically labeled with [32P]orthophosphoric acid and treated with nicotine (1 μM) in the absence or presence of increasing concentrations of propranolol for 60 min. Phosphorylation of Bax was analyzed by Western blot using a Bax antibody. Results revealed that nicotine significantly reduced the half-life ($t_{1/2}$) of Bax in the absence or presence of nicotine (1 μM) for 60 min. The [32P]methionine-labeled cells were washed and incubated in fresh methionine-free RPMI 1640 medium in the absence of presence of nicotine at various times up to 24 h. The half-life ($t_{1/2}$) of Bax was determined by the classical [32P]methionine pulse-chase technique. Results show that nicotine significantly reduced the half-life ($t_{1/2} < 6$ h) of Bax compared with control ($t_{1/2} = 9–12$ h, Fig. 8C). These data indicate that nicotine-induced Bax phosphorylation may reduce the stability of Bax, suggesting another mechanism by which phosphorylation reduces the pro-apoptotic activity of Bax.

**Fig. 7.** Phosphorylation of Bax at Ser-184 results in retention of Bax in cytosol. The GFP-tagged WT, S184A, and S184E Bax mutants were transfected into H157 cells using LipofectamineTM 2000. Subcellular fractionation was performed to isolate mitochondria and cytosol from cells expressing GFP-tagged WT, S184A, or S184E mutant Bax. Bax protein of the mitochondrial (Mito) or cytosolic (Cyto) fraction was analyzed by Western blot using a GFP antibody and confirmed using a Bax antibody.

**Table 1.** Western blot analysis of Bax in various lung cancer cell lines.

| Cell Line   | Mito (%) | Cyto (%) |
|-------------|----------|----------|
| A549        | 75±5     | 25±5     |
| S184A       | 60±3     | 40±3     |
| S184E       | 45±2     | 55±2     |

**Discussion**

Cigarette smoking is by far the most important risk factor in the development of lung cancer. For example, cigarette smokers have a 20-fold higher relative risk of developing lung cancer compared with nonsmokers (1). Ninety percent of all lung cancers are caused by smoking (41). Nicotine is a major component in cigarette smoke that can activate survival signaling pathways and enhance chemoresistance of lung cancer (9). However, our understanding of the molecular mechanism(s) involved is fragmentary. We discovered previously that nicotine induces Bcl2 phosphorylation in association with increased survival of H69 cells expressing high levels of endogenous Bcl2 (42). Unfortunately, most lung cancer cell lines, including A549 cells, do not express detectable levels of Bcl2 but do express high levels of endogenous Bax (Fig. 1A). This suggests that Bax may play a more important role than Bcl2 in nicotine-induced survival of most human lung cancer cells. We discovered that nicotine can potently induce phosphorylation of endogenous Bax in association with prolonged survival of A549 lung cancer cells (Fig. 1A). Other lung cancer cell lines (i.e., H69 and H1299) were also tested, and similar results were obtained (data not shown), indicating that nicotine can induce Bax phosphorylation and survival in various lung cancer cells. Genetic studies indicate that nicotine stimulates Bax phosphorylation exclusively at Ser-184 because only WT, but not the S184A Bax mutant, can be phosphorylated when cells are exposed to nicotine (Fig. 2A). The nonphosphorylatable S184A Bax mutant displays more potent cytotoxic activity than WT Bax. Nicotine can prolong the survival of cells expressing WT but not the S184A Bax mutant. This suggests that the nonphosphorylatable S184A mutant is the active form of Bax and that nicotine is not able to inactivate its proapoptotic function via phosphorylation at the Ser-184 site. By contrast, a substitution of
Ser-184 with glutamate, which mimics Ser-184 site phosphorylation in the hydrophobic transmembrane domain, completely abolishes the proapoptotic activity of Bax (Fig. 2). These findings provide strong evidence that nicotine-induced Bax phosphorylation at Ser-184 may result in a conformational change in the transmembrane domain, which inactivates the proapoptotic function of Bax.

AKT is one of the key enzymes involved in supporting cell survival and can be activated by a variety of growth factors via PI3K-generated phosphatidylinositols. Active AKT is thought to inhibit apoptosis through a mechanism involving both up-stream and downstream mitochondrial perturbation (29). A recent report indicates that PI3K/AKT can suppress the pro-apoptotic activity of Bax by retaining Bax in the cytosol (43). However, the signal mechanism(s) remains unclear. AKT extensively co-localizes with Bax in the cytoplasm, nicotine induces activation of AKT, the purified active AKT can directly phosphorylate either endogenous or recombinant Bax (Fig. 3), and treatment of cells with the PI3K/AKT inhibitor LY294002 or specific knockdown of AKT expression by RNAi blocks both nicotine-induced Bax phosphorylation and survival (Figs. 4 and 5). These findings reveal that AKT functions as a physiological nicotine-activated Bax kinase and provide insight into a previous report demonstrating that GM-CSF stimulates Bax phosphorylation through the PI3K/AKT pathway in neutrophils (29). The phosphomimetic S184E Bax mutant fails to target mitochondrial membranes (28–29) (Fig. 7) in association with loss of its proapoptotic activity (Fig. 2), and these findings help to explain why activation of PI3K/AKT is essential for retention of Bax in the cytosol as well as cell survival (43). AKT-induced
Bax phosphorylation is a likely mechanism by which Bax is retained in the cytosol.

High levels of β-adrenergic receptor are expressed in pulmonary adenocarcinoma cells (44). Mounting evidence now indicates that nicotine can function as a β-adrenergic receptor agonist and that its effect is abrogated by propranolol (a β-adrenergic receptor inhibitor) (13, 40). Because propranolol can block nicotine-induced Bax phosphorylation in A549 cells (Fig. 6) but α-bungarotoxin (α7nAChR specific inhibitor) has no effect (data not shown), this suggests that the β-adrenergic receptor may be the major upstream receptor for nicotine-stimulated Bax phosphorylation in A549 cells. Importantly, inhibition of nicotine-induced Bax phosphorylation by propranolol restores the proapoptotic function of Bax and results in apoptotic cell death (Fig. 6). Thus, propranolol may have the potential to be developed as a clinically useful drug that specifically targets the β-adrenergic receptor to block the nicotine-stimulated survival signal pathway in patients with lung cancer expressing high levels of β-adrenergic receptor and Bax.

Bax is located in the cytosol and only associates peripherally with mitochondrial membranes during normal cell growth but inserts into mitochondrial membranes after a death signal, which results in mitochondrial dysfunction and apoptosis (21). Insertion of Bax into the mitochondria is essential for the proapoptotic activity of Bax (28). Nicotine induces Bax phosphorylation at Ser-184 in the C-terminal hydrophobic tail (Fig. 2), which is critical for Bax to insert into the mitochondrial membranes (28). Because treatment of cells with nicotine not only inhibits stress-induced Bax translocation to mitochondrial membranes but also blocks insertion of Bax into mitochondrial membranes (Fig. 8), nicotine-stimulated Bax phosphorylation may induce a conformational change in its C-terminal hydrophobic tail, which may result in the failure of Bax to target and insert into mitochondrial membranes. Genetic studies further demonstrate that the nonphosphorylatable S184A Bax mutant associates more efficiently with mitochondrial membranes and exhibits more cytotoxic activity than WT Bax. By contrast, the phosphomimetic S184E mutant Bax fails to target mitochondria and presents no proapoptotic activity (Figs. 2 and 7). These findings uncover a novel mechanism by which Bax phosphorylation inactivates its proapoptotic function.

It is now clear that proteasome-mediated degradation plays a crucial role in regulating the expression levels of various regulators that control fundamental cell functions, including both cell cycle and cell death (45–47). Recent studies indicate that Bax is a direct target in an ATP/ubiquitin/proteasome-dependent degradation pathway in various tumor cells (48). Thus, the stability of Bax to proteolysis plays an important role in regulating its proapoptotic activity. Our findings reveal that nicotine significantly reduces the half-life of Bax protein (Fig. 10). Nicotine activates the PI3K/AKT through the upstream β-adrenergic receptor, which can induce Bax phosphorylation at the Ser-184 site in the C-terminal hydrophobic transmembrane domain. The phosphorylated Bax is an inactive form of Bax, which not only fails to insert into mitochondrial membranes but also is more sensitive to proteolytic degradation. Thus, nicotine-induced Bax phosphorylation may sequester Bax from mitochondrial membranes and destabilize Bax protein to abolish the proapoptotic activity of Bax, which may contribute to the development and/or chemoresistance of human lung cancer cells.

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