Protein Kinase Cζ Abrogates the Proapoptotic Function of Bax through Phosphorylation*

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Protein kinase Cζ (PKCζ) is an atypical PKC isoform that plays an important role in supporting cell survival but the mechanism(s) involved is not fully understood. Bax is a major member of the Bcl-2 family that is required for apoptotic cell death. Because Bax is extensively co-expressed with PKCζ in both small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) cells, it is possible that Bax may act as the downstream target of PKCζ in regulating survival and chemosensitivity of lung cancer cells. Here we discovered that treatment of cells with nicotine not only enhances PKCζ activity but also results in Bax phosphorylation and prolonged cell survival, which is suppressed by a PKCζ-specific inhibitor (a myristoylated PKCζ pseudosubstrate peptide). Purified, active PKCζ directly phosphorylates Bax in vitro. Overexpression of wild type or the constitutively active A119D but not the dominant negative K281W PKCζ mutant results in Bax phosphorylation at serine 184. PKCζ co-localizes and interacts with Bax at the BH3 domain. Specific depletion of PKCζ by RNA interference blocks nicotine-stimulated Bax phosphorylation and enhances apoptotic cell death. Intriguingly, forced expression of wild type or A119D but not K281W PKCζ mutant results in accumulation of Bax in cytoplasm and prevents Bax from undergoing a conformational change with prolonged cell survival. Purified PKCζ can directly dissociate Bax from isolated mitochondria of C2-ceramide-treated cells. Thus, PKCζ may function as a physiological Bax kinase to directly phosphorylate and interact with Bax, which leads to sequestration of Bax in cytoplasm and abrogation of the proapoptotic function of Bax.

Apoptosis through the mitochondrial pathway is mainly controlled and mediated by Bcl2 family proteins. Bax is a major multidomain proapoptotic member of the Bcl2 family that is required for apoptotic cell death (1). However, the signaling mechanism(s) by which Bax is regulated remains enigmatic. It has been proposed that activation of the proapoptotic function of Bax likely occurs through several interdependent mechanisms including translocation from cytosol to mitochondria (2), oligomerization, and insertion into mitochondrial membranes following stress (3–5). Recent reports indicate that the proapoptotic activity of Bax can also be regulated by phosphorylation, a post-translational modification (6–9). Growth factor (i.e. granulocyte macrophage-colony-stimulating factor) or survival agonist (i.e. nicotine)-induced Bax phosphorylation at Ser184 through activation of AKT potently suppresses the proapoptotic activity of Bax and prolongs cell survival (6, 9). In contrast, c-Jun NH2-terminal kinase (JNK)-induced Thr167 or glycogen synthase kinase-induced Thr165 phosphorylation of Bax may enhance the proapoptotic activity of Bax (7–8). Intriguingly, we recently discovered that protein phosphatase 2A functions as a physiological Bax phosphatase that not only dephosphorylates Bax but also potently activates its proapoptotic function (10).

The protein kinase C (PKC)2 family is a multigene family that can be subclassified into three groups including classical, novel, and atypical PKC isoforms according to differences in the lipid activation profile. The mechanisms for activation have been established for sequential phosphorylation, the recruiting of proper localization, and the exchanging of binding proteins (11). Growing evidence indicates that PKC family members play important roles in regulating cell survival and apoptosis (12–15). For example, the classic PKCα-induced Bcl-2 phosphorylation enhances antiapoptotic function of Bcl2 in association with increased chemoresistance of human leukemia cells (16). Direct interaction between PKCε and Bax results in suppression of the proapoptotic activity of Bax (17). Bad phosphorylation induced by either PKCζ or PKCθ leads to inactivation of Bad (18, 19). All these findings support the notion that Bcl-2 family members potentially function as downstream targets of PKCs in regulating cell survival and cell death.

PKCζ is a member of the atypical PKC subfamily and plays a critical role in suppression of mitochondrial-dependent apoptosis (20, 21). PKCζ consists of four functional domains and motifs, including a PBD domain in the N terminus, a pseudosubstrate (PS) sequence, a C1 domain of a single Cys-rich zinc finger motif, and a kinase domain in the C terminus.

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2 The abbreviations used are: PKC, protein kinase C; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; BH, Bcl2 homolog domain; PBS, phosphate-buffered saline; DN, dominant negative; WT, wild type; HA, hemagglutinin; PI3K, phosphatidylinositol 3′-OH kinase; siRNA, small interfering RNA; MYR-PKCζ-PS, myristoylated PKCζ pseudosubstrate peptide; MBP, myelin basic protein; PI3P, phosphatidylinositol 1,4,5-trisphosphate; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; MEF, mouse embryonic fibroblast; PKD1, phosphoinositide-dependent kinase.

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(22). The kinase domain includes an ATP-binding region, an activation loop, a turn motif, and a hydrophobic motif. The ATP-binding domain contains a Lys residue (Lys280) that is crucial for its kinase activity. A mutation of Lys→Trp at Lys280 site resulted in a kinase-defective dominant negative form of PKCζ (i.e. K281W) (23). The activation loop and turn motif contain two important phosphorylation sites (i.e. Thr410 and Thr560). Phosphorylation of Thr410 and Thr560 residues is essential for PKCζ activation (24, 25). PKCζ is insensitive to second messengers such as Ca2+ and diacylglycerol. Thus, its activity is primarily regulated by protein-protein interaction and phosphorylation (11). The signal mechanisms for PKCζ activation include the PDK1-dependent (i.e. PI3K/PIP3/PDK1/ PKCζ) and PDK1-independent (i.e. PI3K/PIP3/PKCζ) pathways (22). Growth factor or agonist-induced activation of PI3K produces PIP3. On the one hand, PDK1 binds to PIP3 via its PH domain, which results in activation of PKD1. The activated PDK1 interacts with PKCζ and phosphorylates the kinase domain at Thr410, which induces Thr560 phosphorylation. On the other hand, PKCζ directly interacts with PIP3, which releases PS-dependent autoinhibition. Both contributions of PIP3 and PDK1 are necessary for the complete and stable activation of PKCζ (22). Furthermore, phospholipase D2 has recently been identified as a novel protein cofactor for activation of PKCζ that can enhance PKCζ activity through direct interaction in a lipase activity-independent manner (11). Our previous findings reveal that nicotine stimulates Bax phosphorylation in association with increased cell survival (9). However, a direct signal link between PKCζ and Bax in nicotine-induced cancer cell survival has not been established. Here we have demonstrated that PKCζ functions as a novel nicotine-activated Bax kinase that directly phosphorylates and inactivates Bax in human lung cancer cells.

EXPERIMENTAL PROCEDURES

Materials—Nicotine and cisplatin were purchased from Sigma. Purified, active PKCζ, myristoylated PKCζ pseudosubstrate peptide (MYR-PKCζ-PS, Myr-SIYRRGARRWRKL-OH) and the myristoylated PKCa pseudosubstrate peptide (MYR-PKCa-PS, Myr-N-FARKGLRQ-NH2) were obtained from Calbiochem. Bax, Bcl2, prohibitin, and PKCζ antibodies, PKCζ siRNA1 (sense strand sequence: CAAGCAGAGCUCUAACATT) and PKCζ siRNA2 (sense strand sequence: CAGAUGGAAUUGCUUACAUUtt) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Myelin basic protein (MBP) was obtained from Invitrogen. HA-tagged wild type (WT), constitutively active A119D and dominant negative (DN) K281W PKCζ mutants in pcDNA3 were kindly provided by Dr. Brian Law (University of Florida). Recombinant purified WT, BH1, BH2, and BH3 deletion mutant proteins were purchased from Protein X (San Diego, CA). WT and Bax−/− MEF cells were kindly provided by Dr. Douglas R. Green (St. Jude Children's Research Hospital, Memphis). All reagents used were obtained from commercial sources unless otherwise stated.

Cell Culture, cDNA Constructs, and Transfections—WT and Bax−/− MEF cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 4 mM l-glutamine as described (26). H23, H69, H82, H157, H358, H460, and H1299 were maintained in RPMI1640 with 10% fetal bovine serum. A549 cells were maintained in F-12K medium with 10% fetal bovine serum and 4 mM l-glutamine. For generation of the phosphomimetic and the nonphosphorylatable Bax mutants, the 5’-phosphorylated mutagenic primers for various precise deletion mutants were synthesized as follow: S184A, 5’-GTC- CCTACGGCGCCGTCACTGTTG-3’; S184E, 5’-GTC- CCTACGGCGAGCTCACCCT CGG-3’. The T7-tagged WT-Bax/pUC19 construct was used as the target plasmid that contains a unique NdeI restriction site for selection against the unmutated plasmid. The NdeI selection primer is: 5’-GAGTG- CACCAATGGCGGTGTGAA-3’. These Bax mutants were created using a site-directed mutagenesis kit (Clontech) according to the manufacturer’s instructions. Each single mutant was confirmed by sequencing of the cDNA and then cloned into the pCIneo (Promega) mammalian expression vector. The pCIneo plasmid containing each Bax mutant cDNA was transfected into MEF Bax−/− cells using Lipofectamine™ 2000 according to the manufacturer’s instructions (Invitrogen). The expression levels of exogenous Bax were determined by Western blot analysis using a Bax antibody.

Preparation of Total Cell Lysate—Cells were washed with 1× PBS and resuspended in ice-cold 1% CHAPS lysis buffer (1% CHAPS, 50 mM Tris, pH 7.6, 120 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 50 mM NaF, and 1 mM β-mercaptoethanol) with a mixture of protease inhibitors (Calbiochem). Cells were lysed by sonication and centrifuged at 14,000 × g for 10 min at 4 °C. The resulting supernatant was collected as the total cell lysate and used for protein analysis or co-immunoprecipitation.

Assay of PKCζ Activity in Vitro—PKCζ activity was measured using MBP as substrate as previously described (27). Briefly, PKCζ was immunoprecipitated from cell lysates with an agarose-conjugated PKCζ antibody. Immunoprecipitated PKCζ was washed and resuspended in kinase buffer containing 50 mM Heps (pH 7.5), 100 mM NaCl, 10 mM MgCl2, 50 mM NaF, 1 mM Na3VO4, 1 mM dithiothreitol, and 0.1% Tween 20, 40 μg/ml phosphatidylserine, and 2 μCi of [γ-32P]ATP and MBP (10 μg). The reactions were incubated at 30 °C for 15 min and terminated by addition of SDS sample buffer and boiling the sample for 5 min. The samples were subjected to 12% SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to Kodak X-Omat film at −80 °C. The activity of PKCζ was determined by autoradiography. PKCζ activity was also assessed by measuring the rate of 32P from [γ-32P]ATP incorporation into a peptide (ERMRPRKRGQSVRRV) corresponding to the pseudosubstrate region of PKCζ in which an alanine was replaced by a serine as described (28, 29). Briefly, A549 cells were treated with nicotine for 60 min. Specific immunoprecipitation of PKCζ was performed by incubating a polyclonal PKCζ antibody overnight at 0–4 °C with 1 mg of total cell lysate protein in buffer containing 20 mM Tris/HCl (pH 7.5), 0.25 mM sucrose, 1.2 mM EDTA, 20 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml aprotonin, 1 mM sodium vanadate, 1 mM sodium pyrophosphate, 1 mM NaF, Triton X-100 (1%), Nonidet (0.5%), and 150 mM NaCl. Precipitates using non-immune serum were simultaneously prepared to determine blank values. Precipitates were collected on protein A-Sepharose beads, washed, and suspended in 50
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MM Tris/HCl (pH 7.5), 1 MM NaHCO3, 5 MM MgCl2, 1 MM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 40 μM modified PKCζ pseudosubstrate peptide for 10 min at 30 °C. After thermal equilibration, assays were started by the simultaneous addition of 15 μM ATP, 0.3 μCi of \( \gamma^32P \)ATP (3000 Ci/mmol), and terminated after 30 min with 100 μl of 175 μM phosphoric acid. Then, 100 μl was transferred to P18 filter paper, and washed three times with 75 μM phosphoric acid. The level of peptide phosphorylation was determined by scintillation counting.

Metabolic Labeling, Immunoprecipitation, and Western Blot Analysis—Cells were washed with phosphate-free RPMI medium and metabolically labeled with \( ^32P \)orthophosphoric acid for 120 min. After agonist or inhibitor addition, cells were washed with ice-cold 1 × PBS and lysed in detergent buffer. Bax was immunoprecipitated using an agarose-conjugated Bax antibody. The samples were subjected to 12% SDS-PAGE, transferred to a nitrocellulose membrane, 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 Openlab 3.1.5 software from Improvision, Inc. (Lexington, MA). Areas of protein co-localization appear yellow.

RESULTS

Nicotine Induces Activation of PKCζ and Phosphorylation of Bax in Association with Prolonged Survival of Human Lung Cancer Cells, Which Is Suppressed by a PKCζ-specific Pseudosubstrate Inhibitor—PKCζ has been implicated in many key cellular functions including cell survival (20, 32). Because both human small cell (SCLC) cells (i.e. H69 and H82) and non-small cell lung cancer (NSCLC) cells (i.e. H23, H157, H358, H460, H1299, and A549) express high levels of endogenous PKCζ (Fig. 1A), nicotine may activate PKCζ to promote cell survival. To test this, A549 cells were treated with increasing concentrations of nicotine for 60 min and PKCζ was then immunoprecipitated using an agarose-conjugated PKCζ antibody. Activity of PKCζ was measured by an immune complex kinase assay using purified MBP as a substrate as described (27). The same filter was then probed by Western blotting using a PKCζ antibody. Bax phosphorylation or cell viability was assessed following various treatments. Specific silencing of the targeted PKCζ gene was confirmed by at least three independent experiments.

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FIGURE 1. Nicotine stimulates activation of PKCζ and Bax phosphorylation in association with enhanced survival of human lung cancer cells, which is blocked by a PKCζ-specific inhibitor. A, expression levels of endogenous Bax, Bo2, and PKCζ in various human lung cancer cells were analyzed by Western blot. B, A549 cells were treated with increasing concentrations of nicotine for 60 min. PKCζ was immunoprecipitated (IP) and incubated with purified MBP in an in vitro kinase assay. PKCζ activity was analyzed by autoradiography. PKCζ protein was quantified by Western blot using a PKCζ antibody. To confirm the specificity of the PKCζ antibody, the same filter was reprobed using a PKCζ antibody, respectively. Total cell lysate was used as a positive control for PKCζ, PKC0, or PKCα Western blot. C, PKCζ activity was also measured using a modified PKCζ pseudosubstrate peptide with an Ala to Ser mutation as a PKCζ substrate for the PKCζ activity assay as described (28, 29). Previous reports have demonstrated that a higher level of phosphorylation by PKCζ was obtained with the peptide derived from the pseudosubstrate region of PKCζ compared with the peptide derived from the pseudosubstrate region of PKCζ (28, 29), indicating that the PKCζ pseudosubstrate peptide is a better substrate for PKCζ. Therefore, we chose a modified PKCζ pseudosubstrate peptide (ERMR-PKRQGSRVRRV) as a substrate for the PKCζ activity assay as previously reported (28, 29). Results indicate that treatment of A549 cells with nicotine markedly up-regulates PKCζ activity (Fig. 1C), which is consistent with the results obtained by using MBP as a substrate (Fig. 1B). We have previously demonstrated that nicotine can stimulate Bax phosphorylation in association with increased cell survival (9). To assess whether PKCζ is involved in nicotine-induced Bax phosphorylation, A549 cells were exposed to nicotine in the absence or presence of the PKCζ-specific inhibitor (MYR-PKζ-PS, a myristoylated PKCζ pseudosubstrate peptide) or the myristoylated PKζ pseudosubstrate peptide (MYR-PKζa-PS) (20, 33, 34). Results reveal that MYR-PKζ-PS but not MYR-PKζa-PS potently suppresses nicotine-induced Bax phosphorylation as well as nicotine-prolonged cell survival following cisplatin treatment (Fig. 1, D and E). Other cell lines (i.e. H69, H82, H460, and H1299) were also tested and similar results were obtained (data not shown). These findings suggest that Bax may function as a downstream target of nicotine-activated PKCζ in supporting survival of human lung cancer cells.

Purified PKCζ Directly Phosphorylates Bax in Vitro and Overexpression of PKCζ in Cells Results in Bax Phosphorylation at Ser184 in Vivo—To assess a potential direct role for PKCζ as a physiological Bax kinase, subcellular distribution of PKCζ and Bax was examined by immunofluorescent staining. A mouse antibody against human Bax, rabbit polyclonal PKCζ antibody, and fluorescein isothiocyanate-conjugated anti-mouse (green) or rhodamine-conjugated anti-rabbit (red) secondary antibodies were used so that cells could be simultaneously stained without cross-reaction. As shown in Fig. 2A, Bax is primarily localized with PKCζ in the cytoplasm of A549 cells. To test whether PKCζ can directly phosphorylate endogenous Bax, endogenous Bax was immunoprecipitated from A549 cells and incubated with purified, active PKCζ in a kinase assay buffer containing [γ-32P]ATP. Results indicate that active PKCζ directly phosphorylates endogenous Bax in vitro (Fig. 2B). In addition, active PKCζ can also directly phosphorylate recombinant Bax protein (Fig. 2C). These findings suggest that PKCζ is a strong candidate for being the direct Bax kinase. To determine whether PKCζ may be a Bax kinase, HA-tagged WT, constitutively active A119D, or DN-K281W PKCζ constructs were transfected into A549 cells that express high levels of endogenous Bax. After transfection for 48 h, cells were metabolically labeled with [32P]orthophosphoric acid for 90 min. Bax phosphorylation was analyzed by autoradiography. Results reveal that overexpression of WT or active but not DN-PKCζ results in an increased Bax phosphorylation (Fig. 2D). Importantly, the constitutive active PKCζ has a more potent effect on
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Bax phosphorylation compared with WT (Fig. 2D). To identify the site of Bax phosphorylation induced by PKCζ, WT, S184A, and S184E Bax mutants were co-transfected with the constitutively active A119D PKCζ mutant into MEF Bax−/− cells. After 48 h, cells were then metabolically labeled with [32P]orthophosphoric acid for 90 min. Results reveal that PKCζ induces Bax phosphorylation exclusively at the Ser184 site because expression of active PKCζ results in phosphorylation of WT but not S184A or S184E Bax mutants (Fig. 2F). These findings provide both biochemical and genetic evidence that Bax is a physiological PKCζ downstream substrate. To directly test a role of PKCζ in regulating the proapoptotic function of Bax, a constitutively active PKCζ was co-transfected with WT or each of the S184A and S184E Bax mutants into MEF Bax−/− cells. Consistent with our previous findings (9), the nonphosphorylatable S184A Bax mutant has more proapoptotic activity than WT Bax, whereas the phosphomimetic S184E Bax mutant displays no cytotoxicity (Fig. 2, F and G). Intriguingly, expression of active PKCζ enhances survival of MEF Bax−/− cells expressing exogenous WT Bax but has no significant effect on survival of MEF Bax−/− cells expressing the exogenous S184A or S184E Bax mutant (Fig. 2, F and G). These results indicate that active PKCζ may abolish the proapoptotic function of WT Bax via phosphorylation but fails to reverse the proapoptotic function of the non-phosphorylatable, constitutively active S184A Bax mutant.

Expression of WT or Constitutively Active But Not Dominant Negative PKCζ Results in Retention of Bax in Cytosol and Prevents Bax from Undergoing a Conformational Change—Bax is located in the cytosol and/or peripherally associated with mitochondrial membranes in unstimulated cells (2). To test whether PKCζ-induced Bax phosphorylation affects Bax subcellular localization, WT, active, or DN-PKCζ were transfected into A549 cells. After 48 h, mitochondria and cytosol were isolated and Bax was analyzed using a Bax antibody. Results reveal that overexpression of WT or active PKCζ leads to increased sequestration of Bax in cytosol, whereas expression of DN-PKCζ results in Bax accumulation in mitochondrial membranes (Fig. 3A). To determine the purity of the subcellular fractions obtained, fraction-specific proteins were assessed by probing the same filters. Prohibitin, an exclusively mitochondrial protein (30), was detected only in the mitochondrial fraction, whereas caspase 3, which is a cytosolic protease in growing cells (31), was detected exclusively in the cytosol (Fig. 3A). This indicates that mitochondrial and cytosolic fractions are highly pure without cross-contamination. The monoclonal antibody 6A7, raised against the peptide amino acids 13–19 in the N

![FIGURE 2. PKCζ co-localizes with Bax and directly phosphorylates Bax at the Ser184 site.](image-url)
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terminus of Bax, is not able to bind the soluble form of Bax in healthy cells but can recognize Bax after the conformational change associated with membrane insertion occurs in apoptotic cells (35–37). To test whether expression of PKCζ affects the stress-induced conformational change of Bax, A549 cells expressing constitutively active or DN-PKCζ or vector-only control were treated with cisplatin (40 μM) for 48 h. Antibody binding to Bax was measured by immunofluorescence or immunoprecipitation. Immunoprecipitation of Bax was performed using a 6A7 or pan-Bax antibody and Bax was analyzed by Western blotting using a Bax antibody. Results indicate that treatment of cells with cisplatin potently enhances the ability of the 6A7 antibody to immunoprecipitate Bax compared with the pan-Bax antibody (Fig. 3B, lane 1 versus lane 2). Overexpression of active PKCζ reduces the ability of the 6A7 antibody to immunoprecipitate Bax. By contrast, expression of DN-PKCζ enhances the 6A7 antibody-Bax binding (Fig. 3B). Consistently, Bax immunofluorescence is low or undetectable in untreated vector-only cells, it increases significantly in vector-only cells treated with cisplatin, which is associated with increased apoptotic cell death (Fig. 3, C and D), suggesting that cisplatin is able to induce a conformational change of Bax. Importantly, overexpression of the constitutive active but not DN-PKCζ suppresses the cisplatin-induced Bax conformational change in association with prolonged cell survival (Fig. 3, B–D). Thus, PKCζ-mediated Bax phosphorylation may prevent a conformational change in Bax and retain Bax in an inactive form.

Treatment of Cells with Nicotine Results in Increased PKCζ/Bax Interaction and Decreased Bcl2/Bax Binding, and Purified PKCζ Can Directly Disrupt Bcl2/Bax Heterodimeric Complex in Vitro—Because nicotine can activate PKCζ to phosphorylate Bax (Figs. 1 and 2), it is possible that nicotine-induced activation of PKCζ may facilitate PKCζ to associate with Bax, which may be involved in functionally regulating Bax/Bcl2 heterodimerization. To test this, H460 cells expressing high levels of endogenous PKCζ, Bax, and Bcl2 were treated with increasing concentrations of nicotine for 60 min. PKCζ/Bax and Bax/Bcl2 complexes were immunoprecipitated using a PKCζ or a Bax antibody, respectively. Results indicate that nicotine stimulates PKCζ to interact with Bax but not Bcl2 in a dose-dependent manner (Fig. 4A, upper panel). Intriguingly, nicotine-stimulated PKCζ/Bax association results in decreased Bcl2/Bax binding (Fig. 4A, lower panel). To test whether PKCζ can directly affect Bcl2/Bax heterodimeric complex in vitro, the Bcl2-Bax complex was immunoprecipitated from the lysate of H460 cells disrupted in 1% CHAPS lysis buffer using an agarose-conjugated Bax antibody. The immune complex was incubated with purified, active PKCζ in a kinase buffer at 30 °C for 30 min and proteins released from the complex were identified in the supernatant following centrifugation at 14,000 × g for 5 min. Bax-associated PKCζ (i.e. bound PKCζ), Bax-associated monoclonal 6A7 primary and fluorescein isothiocyanate-conjugated mouse secondary (green) antibodies. Images were merged using Openlab 3.1.5 software. Areas of co-localization are yellow. D, A549 cells expressing active, DN-PKζ or vector-only control were treated with cisplatin (40 μM) for 48 h. Cell viability was assessed as described in the legend for Fig. 1E. Data represent the mean ± S.D. of three separate determinations.
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**A**

![Graph A](image)

**B**

![Graph B](image)

Bcl2 (i.e., bound Bcl2), unbound Bcl2 (i.e., present in the supernatant), and total Bax were analyzed by Western blot. Results reveal that PKCζ can directly disrupt Bcl2/Bax in vitro because decreased levels of Bcl2 were observed on beads and increased levels of Bcl2 were present in the supernatant (Fig. 4B). These findings suggest that PKCζ can directly disrupt the Bax/Bcl2 heterodimer via its binding to Bax, which results in liberation of Bcl2 to benefit the antiapoptotic function of Bcl2.

Active But Not DN-PKCζ Associates with Bax at Its BH3 Domain—To test whether the activity of PKCζ affects its ability to interact with Bax, HA-tagged WT, active or DN-PKCζ were transfected into A549 cells using Lipofectamine® 2000. Co-immunoprecipitation using a monoclonal HA antibody indicated that WT and active but not DN-PKCζ can interact with Bax (Fig. 5A, upper panel). Reciprocally, Bax associates with both WT and the active PKCζ but fails to interact with the DN-PKCζ mutant (Fig. 5A, lower panel). Importantly, the active PKCζ more efficiently binds to Bax compared with WT (Fig. 5A). Because the DN-PKCζ mutant resulting from a Lys → Trp mutation at Lys281 in the ATP-binding domain has no ability to interact with Bax (Fig. 5A), this suggests that a functional ATP-binding domain is critical for PKCζ to associate with Bax. Bax contains the Bcl2 homology (BH) domains including BH1, BH2, and BH3 (38). To directly assess whether PKCζ binds to Bax at these BH domains, purified PKCζ was incubated with purified recombinant WT, ΔBH1, ΔBH2, or ΔBH3 Bax deletion mutants in 1% CHAPS lysis buffer at 4 °C for 2 h. The PKCζ-associated Bax was co-immunoprecipitated with PKCζ antibody and analyzed by Western blot.

**FIGURE 5.** PKCζ directly interacts with Bax at the BH3 domain. A, the pcDNA3 plasmids bearing HA-tagged WT, constitutively active A119D, and DN-K281W PKCζ mutants were transfected into A549 cells that express high levels of endogenous Bax using Lipofectamine 2000. After 48 h, a co-immunoprecipitation (IP) experiment was carried out using HA or Bax antibody, respectively. The PKCζ-associated Bax or Bax-associated PKCζ were analyzed by Western blot using Bax or HA antibody, respectively. B, purified PKCζ (0.1 μg) was incubated with purified WT, ΔBH1, ΔBH2, or ΔBH3 Bax deletion mutants (0.1 μg each) in 1% CHAPS lysis buffer at 4 °C for 1 h. The PKCζ-associated Bax was co-immunoprecipitated with PKCζ antibody and analyzed by Western blot.
whether Bax protein slightly associates with or inserts into mitochondrial membranes (2, 10). To test whether PKCζ can induce phosphorylation of the mitochondrial inserted Bax, A549 cells were treated with C2-ceramide for 24 h. Mitochondria were isolated and incubated in 0.1 M Na2CO3 (pH 11.5) on ice for 30 min, and centrifuged at 200,000 × g to yield a mitochondrial pellet. The resulting alkali-extracted mitochondrial membrane pellet was washed three times with a kinase buffer, and incubated with purified, active PKCζ in the kinase assay buffer containing [γ-32P]. Phosphorylation of Bax was determined by autoradiography. Depletion of PKCζ expression. In contrast, PKCa siRNA can specifically knock down PKCa but has no effect on PKCζ expression (Fig. 7A). These data reveal that the effect of PKCζ siRNA on PKCζ expression is specific and is not a consequence resulting from off-target effects. Functionally, specific disruption of PKCζ expression by either PKCζ siRNA1 or siRNA2 but not by control siRNA or PKCa siRNA suppresses nicotine-induced Bax phosphorylation as well as nicotine-stimulated survival of human lung cancer cells (Fig. 7). These findings support the notion that PKCζ may be essential for nicotine-induced Bax phosphorylation to positively regulate lung cancer cell survival.

**DISCUSSION**

Nicotine has been found to prolong cell survival, which may be associated with increased chemoresistance of human lung cancer cells, but understanding of the molecular mechanism(s) is fragmentary (9, 40). PKC isoforms that appear to be anti-apoptotic include PKCa, PKC βII, PKCe, PKCa, and PKCζ (41). Previous studies reveal that the drug-resistant phenotype is associated with expression and/or activity of PKCs in lung cancer cell lines and lung carcinomas (41). PKCζ is an atypical PKC isoform that is ubiquitously expressed in both SCLC and NSCLC cells (Fig. 1A). Because nicotine can potentiate
PKCζ and enhance survival of human lung cancer cells (Fig. 1), nicotine-stimulated survival and/or chemoresistance may occur, at least in part, through activation of PKCζ. PKCζ has been reported to act as an anti-apoptotic protein kinase (20) but the downstream survival effectors involved have not been fully identified. It is well known that the decision phase for apoptotic cell death is largely regulated by the Bcl-2 family members (42). Thus, Bcl2 family member(s) may be the most attractive candidate for the substrate of PKCζ in nicotine-stimulated survival signaling. Bax, a major multidomain proapoptotic member of the Bcl2 family, is widely expressed in various lung cancer cells. Importantl, nicotine potently induces Bax phosphorylation that can be blocked by a PKCζ specific inhibitor (i.e. MYR-PKCζ-PS) (Fig. 1), suggesting that Bax may function as a downstream survival substrate of PKCζ in human lung cancer cells.

Evidence reported here indicates that PKCζ may be a physiological Bax kinase because PKCζ co-localizes with Bax in the cytoplasm and directly phosphorylates either endogenous or recombinant Bax in vitro, indicating its potential, direct role as a Bax kinase (Fig. 2). Confirmation of PKCζ as a physiological Bax kinase was obtained in vivo from results of transfection studies demonstrating that overexpression of HA-tagged WT or constitutively active-PKCζ but not DN-PKCζ in A549 cells, resulted in enhanced phosphorylation of Bax (Fig. 2D), which supports in vitro results. We have previously discovered that nicotine induces Bax phosphorylation at the Ser184 site and prolongs cell survival (9). Genetic studies further demonstrated that the nonphosphorylatable S184A Bax mutant is the active form that has more proapoptotic activity than WT, whereas the phosphomimetic S184E Bax mutant is an inactive form that has more proapoptotic activity than WT, whereas the nonphosphorylatable S184A Bax mutant is the active form that not only recognizes active, conformationally changed Bax, and apoptotic cell death. These genetic studies reveal that PKCζ-mediated Bax phosphorylation may inhibit the Bax conformational change to retain Bax in an inactive form followed by stress signal.

In addition to Bax phosphorylation, nicotine also stimulates a direct PKCζ/Bax interaction, which leads to dissociation of the Bcl2/Bax heterodimer (Fig. 4A). Intriguingly, the activity of PKCζ is necessary for its binding to Bax because only active PKCζ but not DN-PKCζ has the ability to interact with Bax (Fig. 5). Purified PKCζ can directly disrupt the Bcl2/Bax complex by binding to the BH3 domain of Bax (Figs. 4 and 5). The consequence of this interaction is the release of anti-apoptotic Bcl2 protein from the Bcl2/Bax heterodimer, which may facilitate the formation of the Bcl2 homodimer to enhance the antiapoptotic function of Bcl2. Because the BH3 domain is required for the killing activity of Bax (43, 44), the direct binding of PKCζ to the BH3 domain of Bax may potentially impede Bax to exert its proapoptotic function. Thus, PKCζ may provide a double whammy to the proapoptotic function of Bax through interaction with its BH3 domain and phosphorylation at the Ser184 site.

Mitochondria play a central role in apoptosis that is mainly regulated by Bcl2 family members (45). Bax is a major proapoptotic Bcl2 family member present in the cytosol and/or “peripherally” associates with mitochondrial membranes in an inactive state during normal cell growth. In response to apoptotic stimuli, Bax undergoes specific conformational changes, which allow its targeting/insertion into mitochondrial membranes (46). Our previous findings reveal that C2-ceramide-induced Bax dephosphorylation promotes Bax targeting and insertion into mitochondrial membrane (10). Here we found that treatment of isolated mitochondria from C2-ceramide-treated cells with purified PKCζ in vitro results in dissociation of Bax from isolated mitochondrial membranes (Fig. 6A). Further studies indicate that PKCζ can directly phosphorylate the alkali-resistant, mitochondrial membrane-inserted Bax (Fig. 6B), suggesting that PKCζ-mediated Bax phosphorylation may facilitate extraction of Bax from mitochondrial membranes, which converts Bax from an active form to inactive status. These findings support a notion that PKCζ-mediated suppression of apoptosis may occur through a mechanism by negatively regulating the mitochondrial membrane integration of Bax.

In summary, our findings have identified PKCζ as a novel nicotine-activated protein kinase that can directly phosphorylate the multidomain proapoptotic protein, Bax, at Ser184. PKCζ-induced Bax phosphorylation leads to sequestration of Bax in the cytoplasm, suppression of Bax conformational change, and dissociation of Bax from mitochondrial membranes, which may result in abrogation of the proapoptotic function of Bax. Activated PKCζ can directly interact with Bax at the BH3 domain, and this interaction disrupts the Bcl2/Bax heterodimer to liberate Bcl2, which may potentially impede the proapoptotic function of Bax and benefit the antiapoptotic function of Bcl2. Thus, nicotine-induced survival and chemoresistance of human lung cancer cells may occur in a novel mechanism involving activation of PKCζ that not only phosphorylates but also interacts with Bax to potently inactivate its proapoptotic function.
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