Nicotine Induces Resistance to Chemotherapy by Modulating Mitochondrial Signaling in Lung Cancer

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Continued smoking causes tumor progression and resistance to therapy in lung cancer. Carcinogens possess the ability to block apoptosis, and thus may induce development of cancers and resistance to therapy. Tobacco carcinogens have been studied widely; however, little is known about the agents that inhibit apoptosis, such as nicotine. We determine whether mitochondrial signaling mediates antiapoptotic effects of nicotine in lung cancer. A549 cells were exposed to nicotine (1 μ M) followed by cisplatin (35 μ M) plus etoposide (20 μ M) for 24 hours. We found that nicotine prevented chemotherapy-induced apoptosis, improved cell survival, and caused modest increases in DNA synthesis. Inhibition of mitogen-activated protein kinase (MAPK) and Akt prevented the antiapoptotic effects of nicotine and decreased chemotherapy-induced apoptosis. Small interfering RNA MAPK kinase-1 blocked antiapoptotic effects of nicotine, whereas small interfering RNA MAPK kinase-2 blocked chemotherapy-induced apoptosis. Nicotine prevented chemotherapy-induced reduction in mitochondrial membrane potential and caspase-9 activation. Antiapoptotic effects of nicotine were blocked by mitochondrial anion channel inhibitor, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid. Chemotherapy enhanced translocation of proapoptotic Bax to the mitochondria, whereas nicotine blocked these effects. Nicotine up-regulated Akt-mediated antiapoptotic X-linked inhibitor of apoptosis protein and phosphorylated proapoptotic Bcl2-antagonist of cell death. The A549-p0 cells, which lack mitochondrial DNA, demonstrated partial resistance to chemotherapy-induced apoptosis, but blocked the antiapoptotic effects of nicotine. Accordingly, we provide evidence that nicotine modulates mitochondrial signaling and inhibits chemotherapy-induced apoptosis in lung cancer. The mitochondrial regulation of nicotine imposes an important mechanism that can critically impair the treatment of lung cancer, because many cancer-therapeutic agents induce apoptosis via the mitochondrial death pathway. Strategies aimed at understanding nicotine-mediated signaling may facilitate the development of improved therapies in lung cancer.

Keywords: apoptosis; chemotherapy; lung cancer; mitochondria; nicotine

Accumulating evidence shows that continued smoking increases tumor progression and resistance to cancer therapy in patients with lung cancer (1, 2). Tumor promoters possess the ability to block apoptosis, an important mechanism in the development and growth of tumors, especially if inhibition of apoptosis coincides with or follows exposure to known carcinogens (3–5). Many cancer therapeutic agents, including drugs, radiation, and heat treatments, appear to kill tumor cells by apoptosis (6, 7).

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CLINICAL RELEVANCE

Nicotine induces resistance to chemotherapy-induced apoptosis by modulating mitochondrial signaling. These effects of nicotine are critical in patients undergoing lung cancer treatment, since cancer therapy induces apoptosis via mitochondrial pathway.

Hence, an inhibition of apoptosis may promote resistance to cancer therapy. Environmental tobacco carcinogens have been widely studied; however, little is known about agents that inhibit apoptosis, such as nicotine.

Nicotine is a major tobacco alkaloid, an addictive component of cigarette smoke. High-affinity nicotine acetylcholine receptors (nAChRs) are found on both human lung cancer cells and normal lung cells (8–10). Recent reports show that nicotine inhibits apoptosis in various cells lines, which may suggest that nicotine has the ability not only to promote lung cancer development by activating cell growth pathways, but also to reduce the efficacy of chemotherapeutic agents by stimulating survival pathways (11–14). However, the intracellular mechanisms underlying nicotine-induced survival and chemoresistance of human lung cancer cells remain elusive. We hypothesize that nicotine may induce resistance to cancer therapy by inhibition of apoptosis in lung cancer cells. Because activation of the mitochondrial death pathway is one of the most common mechanisms by which many cancer-therapeutic agents induce apoptosis in tumor cells, we sought to determine whether nicotine prevents activation of the mitochondrial death pathway and inhibits apoptosis in lung cancer cells.

MATERIALS AND METHODS

Materials

Nicotine and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

A549 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine (0.3 μ g/ml), nonessential amino acids, penicillin (100 U/ml), streptomycin (200 μ g/ml), and 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY) in a humidified, 95% air–5% CO₂ incubator at 37°C. Targeting small interfering (si) RNA mitogen-activated protein kinase (MAPK) kinase (MEK)-1 and siRNA MEK2 was done by transfecting A549 cells using commercially available MEK1 siRNA and MEK2 siRNA duplexes (Cell Signaling Lab, Danvers, MA) exactly per the manufacturer's protocol. Down-regulation of MEK1 and MEK2 was assessed by Western blot. After transfection, the cells were pretreated with nicotine, followed by cisplatin and etoposide.

Production and Characterization of A549-p0 Cells Lacking Mitochondrial DNA

A549-p0 cells were generated by slow chemical elimination of mitochondrial DNA by culturing in medium supplemented with ethidium bromide (25 ng/ml), sodium pyruvate (1 mM), and uridine (50 μ g/ml), as previously

described by our group and others (15–17). The lack of normal oxidative phosphorylation in A549-p0 cells was demonstrated by comparing antimycin-A-induced reactive oxygen species (ROS) formation in A549 and A549-p0 cells by using dichlorofluorescein assay (data not shown).

Apoptosis Assay

Alveolar epithelial cell apoptosis was assessed by DNA nucleosomal fragmentation ELISA (Roche Diagnostics, Indianapolis, IN), as previously described (18). The DNA nucleosomal fragmentation ELISA assay detects histone-associated DNA fragments (mono- and oligonucleosomes). These assays are well known to correlate directly with alveolar epithelial cell apoptosis, as assessed by acridine orange-stained nuclear morphology, annexin V staining, and caspase-3 activation.

[³H]Thymidine Incorporation Assay

Cells were incubated with [³H]thymidine (1 μ Ci/ml [³H]-TdR) for 6 hours, then washed and reincubated with 10% trichloroacetic acid solution twice for 5 minutes each at 4°C followed by 10% SDS for 2 minutes at room temperature. The radioactivity was quantified in a scintillation counter.

Disodium Salt Cell Viability Assay

Cells were treated with nicotine with and without chemotherapy and then incubated with 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt (MTT) (20 μ l) for 3 hours. An absorbance at 490 nm was measured to quantify the formazan product.

Mitochondrial Membrane Potential Change

The membrane potential change ($\Psi\Delta m$) was assessed by a fluorometric assay using tetramethylrhodamine ethyl ester (TMRE; Molecular Probes, Eugene, OR) or Mitotracker green (Molecular Probes) followed by carbonyl cyanide trifluoromethoxyphenylhydrazone (FCCP; Sigma), as previously described by our group (18). Briefly, A549 cells were pretreated with nicotine (1 μ M) followed by cisplatin and/or etoposide for 24 hours and then exposed to either TMRE (500 nM) or Mitotracker green (1 μ M) for 1 hour at 37°C. FCCP (20 μ M) was added to a separate group of comparably treated cells for 1 hour before adding fluorochromes to induce a maximal $\Psi\Delta m$ by uncoupling oxidative phosphorylation and eliminating the mitochondrial proton gradient. Changes in dye fluorescence at 25°C were analyzed in a fluorometer using an excitation wavelength of 488 nm and emission wavelengths of 520 or 580 nm (TMRE and Mitotracker green fluorescence, respectively). The Mitotracker green was used to label the mitochondria, because it binds mitochondrial lipids and is not influenced by the $\Psi\Delta m$ caused by FCCP. TMRE is one of the preferred fluorochromes to monitor the $\Psi\Delta m$, because, in the nanomolar range, TMRE exclusively stains the mitochondria and is not retained in cells upon collapse of $\Psi\Delta m$. The $\Psi\Delta m$ was compared qualitatively based upon the percentage difference in the ratio of TMRE and Mitotracker green fluorescence of untreated cells (Te and MGc, respectively), corrected for the background fluorescence in FCCP-treated control cells (FTc and FMGc) and the ratio of TMRE and Mitotracker green fluorescence of treated cells (Tt and Mgi) minus the FCCP-treated cells (FTt and FMGt), respectively defined as follows: $\Psi\Delta m = (Tc/MGc - FTc/MGc) - (Tt/MGt - FTt/FMGt) \times 100$.

TMRE untreated control cells (Tc); TMRE treated cells (Tt); Mitotracker green fluorescence untreated control cells (MGc); Mitotracker green fluorescence of treated cells (MGt); background fluorescence with FCCP in control cells (FTc); background fluorescence with FCCP in treated cells (FTt); background fluorescence with FCCP and Mitotracker green in control cells (FMGc); background fluorescence with FCCP and Mitotracker green in treated cells (FMGt).

Caspase Activity Assays

Caspase-9 activity was assessed by using colorimetric activity assay kits from Upstate Laboratories (East Syracuse, NY) per the manufacturer's protocol.

Western Blot Analysis

Cells were treated, washed, and lysed. Proteins were size fractionated by 10% gel electrophoresis and transferred to nitrocellulose mem-

branes using a semidry transfer (Bio-Rad, Hercules, CA). Blots were incubated with specific antibodies overnight at 4°C and developed with an enhanced chemiluminescence detection kit (Amersham).

Immunofluorescence Staining

Cells were treated with nicotine (1 μ M) followed by cisplatin and etoposide for 24 hours, and then incubated with 1 μ M Mitotracker red to stain the mitochondria. The slides were rinsed, fixed, and then incubated overnight with a specific antibody, Bax (Cell Signaling Technology, Beverly, MA), followed by 4',6-diamidino-2-phenylindole for nuclear staining.

Statistical Analysis

Data are reported as mean $\pm$ SEM. Statistical analysis was done by one-way ANOVA and Tukey's tests. Results were considered significant at $P < 0.05$.

RESULTS

Nicotine Inhibits Cisplatin and Etoposide-Induced Apoptosis in A549 Cells

Combination chemotherapy is an ideal therapeutic approach recommended in patients with lung cancer. Although cisplatin is a common anticancer agent used in lung cancer, some investigators have shown a varying level of cisplatin resistance in lung cancer cells, possibly due to genetic modulation (19). Therefore, we designed a study using a combination of two chemotherapeutic agents to assess antiapoptotic effects of nicotine in A549 cells. A549 cells were treated with nicotine (1 μ M) for 24 hours, followed by cisplatin (35 μ M), etoposide (20 μ M), or both together, and then incubated with 10% FBS for 24 hours. Apoptosis was assessed by DNA nucleosomal fragmentation ELISA (Roche Diagnostics) (Figure 1A) and MTT assay (Figure 1B). DNA nucleosomal fragmentation ELISA is well known to correlate directly with A549 cell apoptosis, as assessed by acridine orange-stained nuclear morphology, annexin V staining, and caspase-3 activation. This assay detects histone-associated DNA fragments (mono- and oligonucleosomes). As shown in Figure 1A, nicotine significantly reduced both cisplatin and etoposide-induced apoptosis as compared with the control in A549 cells. In our dose-response study with nicotine (0.1, 1, and 10 μ M), the maximum protective effect of nicotine against apoptosis was observed at the dose of 1 μ M in A549 cells (data not shown). This dose was chosen for conducting all further experiments. A combination of cisplatin and etoposide caused a 7.5-fold increase in A549 cell apoptosis as compared with cisplatin alone (fourfold) or etoposide alone (fivefold). However, pretreatment of A549 cells with nicotine, in part, prevented these effects. In addition to A549 cells, we also examined the antiapoptotic effects of nicotine in H441 and H23 non-small cell lung cancer cells. As shown in Figure 1C, combination chemotherapy caused an increase in DNA fragmentation ELISA by sixfold in H441 cells and threefold in H23 lung carcinoma cells. However, nicotine prevented chemotherapy-induced apoptosis in each of these lung cancer cells. These data suggest that nicotine plays a central role in the inhibition of chemotherapy-induced apoptosis in lung cancer. Furthermore, using MTT assay, and similar study conditions, we examined effects of nicotine on cell survival in A549 cells. As shown in Figure 1B, nicotine induced cell survival in cells exposed to nicotine with or without cisplatin and etoposide at 48 hours. Collectively, the above data demonstrate an antiapoptotic effect of nicotine against chemotherapy-induced apoptosis in A549 cells.

Nicotine Causes a Modest Increase in A549 Cell DNA Synthesis

A549 cells were pretreated with 1 μ M nicotine for 24 hours, followed by cisplatin and etoposide, and then incubated with 10%

FBS for 24 hours. ^3H -thymidine incorporation assay was used to assess DNA synthesis in A549 cells that are exposed to nicotine, as described in MATERIALS AND METHODS. As shown in Figure 1D, a modest increase in ^3H -thymidine uptake was observed in cells exposed to nicotine with or without cisplatin and etoposide, suggesting that the antiapoptotic effects of nicotine may occur in part by enhanced DNA synthesis in these cells.

Figure 1. Nicotine prevents chemotherapy-induced apoptosis in A549 cells. (A) The cells were treated with nicotine (1 μM) for 24 hours, followed by cisplatin (35 μM), etoposide (20 μM), or both together for 24 hours. The A549 cell apoptosis was assessed by DNA nucleosomal fragmentation ELISA (18). Combination chemotherapy induced a sixfold increase in A549 cell apoptosis, whereas cisplatin or etoposide alone caused 3- to 3.5-fold increase. Pretreatment of A549 cells with nicotine, in part, blocked these effects. Mean $\pm$ SEM, * $P < 0.05$ control versus cisplatin/etoposide/both; † $P < 0.05$ cisplatin/etoposide/both versus nicotine + cisplatin/etoposide/both; $n = 3$. Solid bars, control; shaded bars, nicotine. (B) Solid bars represent no nicotine, and shaded bars represent nicotine. (C) Antiapoptotic effects of nicotine against chemotherapy-induced apoptosis were also seen in A549, H441, and H23 non-small cell lung cancer cells. * $P < 0.05$ control (dotted bars) versus cisplatin + etoposide (open bars); † $P < 0.05$ cisplatin + etoposide versus nicotine + cisplatin + etoposide (hatched bars), $n = 3$. Solid bars, nicotine. (B and D) Nicotine improved cell viability, as assessed by disodium salt assay, and caused modest increases in DNA synthesis as assessed by ^3H -thymidine uptake in A549 cells. * $P < 0.05$ control versus nicotine; † $P < 0.05$ control versus cisplatin/etoposide/both; ‡ $P < 0.05$ cisplatin/etoposide/both versus nicotine + cisplatin/etoposide/both, $n = 3$.

MAPK Pathway, in Part, Mediates the Antiapoptotic Effects of Nicotine as well as Chemotherapy-Induced Apoptosis in A549 Cells

We first determined whether nicotine induces activation of MAPK in A549 cells through Western blot assay. As shown in Figure 2A, nicotine caused up-regulation of phosphorylated extracellular signal-regulated kinase as early as 10 minutes in these cells. The cells were then pretreated with MAPK inhibitor, U0126 (10 μM), followed by nicotine and then cisplatin to determine the role of MAPK in mediating antiapoptotic effects of nicotine. As shown in Figure 2B, U0126 alone caused a slight decrease in cisplatin-induced apoptosis in A549 cells; however, antiapoptotic effects of nicotine were significantly blocked by U0126. These data suggest that the MAPK pathway plays a critical role in both cisplatin-induced apoptosis and in mediating the antiapoptotic effects of nicotine in A549 cells.

MEK1 Mediates Antiapoptotic Effects of Nicotine against Chemotherapy-Induced Apoptosis, whereas MEK2 Mediates Chemotherapy-Induced Apoptosis in A549 Cells

To differentiate the role of MAPK in mediating chemotherapy-induced apoptosis versus the effects of nicotine, we used siRNA MEK1 and siRNA MEK2, and assessed the effects of nicotine in chemotherapy-induced apoptosis by using terminal transferase dUTP-digoxigenin nick end-labeling assay and caspase-9 activation by Western blot in A549 cells. As shown in Figures 2C and 2D, down-regulation of MEK1 blocked antiapoptotic effects of nicotine against chemotherapy-induced apoptosis, whereas down-regulation of MEK2 prevented chemotherapy-induced apoptosis and caspase-9 activation, suggesting a differential role of MAPKs in mediating these effects in A549 cells. Furthermore, inhibition of MAPK by U0126 did not completely block the effects of nicotine and chemotherapy in these cells, suggesting that an alternative mechanism may play a role in mediating these effects (Figure 2B). Therefore, we examined the Akt pathway in these cells (14).

Akt Pathway Plays a Partial Role in Mediating Antiapoptotic Effects of Nicotine as well as Chemotherapy-Induced Apoptosis in A549 Cells

To determine the role of Akt in mediating the effects of nicotine in these cells, we first determined whether nicotine induces activation of Akt in A549 cells. As shown in Figure 2E, exposure

Figure 2. Antipapoptotic effects of nicotine, in part, are regulated by mitogen-activated protein kinase (MAPK)-mediated mitochondrial death pathway and Akt pathway. (A) Nicotine induces activation of MAPK in as early as 10 minutes in A549 cells, as assessed by Western Blot assay. (B) MAPK inhibitor, U0126, alone, slightly decreased cisplatin-induced apoptosis; however, it completely blocked the antipapoptotic effects of nicotine in A549 cells. * $P < 0.05$ control versus cisplatin; † $P < 0.05$ cisplatin versus nicotine + cisplatin; ‡ $P < 0.05$ cisplatin versus U0126 + cisplatin; § $P < 0.05$ versus nicotine + cisplatin versus U0126 + nicotine + cisplatin, $n = 3$. Solid bars, control; hatched bars, nicotine. (C and D) Small interfering (si) RNA MAPK kinase (MEK)-1 prevents the protective effects of nicotine against chemotherapy-induced apoptosis; however, siRNA MEK2 blocks chemotherapy-induced apoptosis by terminal transferase dUTP-digoxigenin nick end-labeling (TUNEL) and caspase-9 activation. (E) Nicotine induces activation of Akt and X-linked inhibitor of apoptosis protein (XIAP) as early as 30 minutes, as assessed by Western Blot assay. (F) Antipapoptotic effects of nicotine, in part, are regulated by MAPK-mediated mitochondrial death pathway and Akt pathway. Addition of MAPK inhibitor, U0126, or Akt inhibitor, LY294002, in part, inhibited chemotherapy-induced apoptosis and blocked the antipapoptotic effects of nicotine in these cells. However, addition of both inhibitors together completely blocked the antipapoptotic effects

of nicotine in these cells. * $P < 0.05$ control (dotted bars) versus cisplatin + etoposide (open bars); † $P < 0.05$ cisplatin + etoposide versus nicotine + cisplatin + etoposide (hatched bars); ‡ $P < 0.05$ cisplatin + etoposide versus U0126 or LY294002 + cisplatin + etoposide; § $P < 0.05$ versus nicotine + cisplatin + etoposide versus U0126 or LY294002 + nicotine + cisplatin + etoposide, $n = 3$. Solid bars, nicotine.

of nicotine caused induction of Akt as early as 30 minutes. Nicotine induced up-regulation of X-linked inhibitor of apoptosis protein (XIAP), a downstream effector protein in the Akt pathway that mediates antipapoptotic effects of nicotine in these cells (14). Cells were then pretreated with Akt inhibitor, LY294002, with and without U0126. The effect of nicotine on combination chemotherapy-induced apoptosis was assessed by using DNA fragmentation ELISA. As shown in Figure 2F, LY294002 caused a slight reduction in chemotherapy-induced apoptosis and blocked the effects of nicotine in these cells. Addition of MAPK inhibitor, U0126, alone caused very significant reduction in cisplatin- and etoposide-induced apoptosis, and blocked the antipapoptotic effects of nicotine. However, addition

of U0126 and LY294002 together completely blocked cisplatin- and etoposide-induced apoptosis, and the protective effects of nicotine in these cells. These data suggest that both MAPK and Akt pathways play a critical role in mediating the effects of nicotine and chemotherapy in lung cancer cells.

Nicotine Modulates Chemotherapy-Induced Apoptosis via the Mitochondrial Death Pathway in A549 Cells

Several cancer-therapeutic agents appear to kill tumor cells by inducing apoptosis, and many of these agents work by activation of the mitochondrial death pathway preventing release of cytochrome c and caspase-9 activation (3–6, 12). Therefore, modulation of mitochondrial death pathway by nicotine was examined at

Figure 2. (continued)

multiple levels in the mitochondrial signaling cascade. The A549 cells were pretreated with pan-caspase inhibitor, N-benzyloxycarbonyl-valyl-alanyl-aspartyl-fluoromethylketone (zVAD; 40 μ M), followed by nicotine (1 μ M), and then cisplatin (35 μ M) and etoposide (20 μ M) together. The cells were then incubated in 10% FBS for 24 hours, and apoptosis and cell survival were assessed by MTT assay. As shown in Figures 3A and 3B, nicotine prevented cisplatin- and etoposide-induced apoptosis; the pan-caspase inhibitor, zVAD, partially blocked cisplatin- and etoposide-induced apoptosis. However, the antiapoptotic effect of nicotine was completely blocked by zVAD in these cells. Addition of Akt inhibitor LY294002 with zVAD completely blocked the apoptotic effect of chemotherapy, as assessed by DNA frag-

mentation ELISA. Furthermore, the cells pretreated with zVAD, LY294002, or both showed higher MTT as compared with the cells treated with chemotherapy alone, suggesting that both inhibitors block chemotherapy-induced apoptosis as well as the effects of nicotine in these cells.

Next, we examined the effects of nicotine on cisplatin-induced caspase-9 activation in A549 cells by Western blot assay. As shown in Figure 3C, cisplatin induced a 2.5-fold increase in caspase-9 activation, whereas nicotine completely blocked cisplatin-induced caspase-9 activation in A549 cells. To determine the effect of nicotine on $\Delta\Psi_m$, A549 cells were treated with nicotine, followed by cisplatin and etoposide, as described above. The $\Delta\Psi_m$ was assessed by a fluorometric technique with TMRE

Figure 3. Nicotine modulates chemotherapy-induced apoptosis via mitochondrial death pathway in A549 cells. (A) Nicotine (solid bars) prevented cisplatin- and etoposide-induced apoptosis. The antiapoptotic effects of nicotine were blocked by the pan-caspase inhibitor, zVAD. Furthermore, zVAD partially blocked cisplatin- and etoposide-induced apoptosis in A549 cells, and an addition of LY294002 Akt inhibitor completely blocked chemotherapy-induced apoptosis and the effects of nicotine in these cells. * $P < 0.05$ control (dotted bars) versus cisplatin + etoposide (open bars); † $P < 0.05$ cisplatin + etoposide versus nicotine + cisplatin + etoposide (hatched bars); ‡ $P < 0.05$ cisplatin + etoposide versus zVAD or zVAD + LY294002 + cisplatin + etoposide, $n = 3$. (B) Inhibition of mitochondrial death pathway prevents combination chemotherapy-induced decreases in cell survival and blocks the effects of nicotine in A549 cells. * $P < 0.05$ control versus cisplatin + etoposide; † $P < 0.05$ cisplatin + etoposide versus nicotine + cisplatin + etoposide; ‡ $P < 0.05$ cisplatin + etoposide versus zVAD or zVAD + LY294002 + cisplatin + etoposide; ¶ $P < 0.05$ nicotine + cisplatin + etoposide versus zVAD or zVAD + LY294002 + nicotine + cisplatin + etoposide, $n = 3$. Solid bars, no nicotine; open bars, nicotine. (C) Nicotine inhibited cisplatin-induced caspase-9 activation in A549 cells. Cisplatin induced a 2.5-fold increase in caspase-9, whereas nicotine completely blocked these effects. * $P < 0.05$ control versus cisplatin; † $P < 0.05$ cisplatin versus nicotine + cisplatin, $n = 3$. (D) Nicotine prevents chemotherapy-induced mitochondrial dysfunction by inhibiting reduction in mitochondrial membrane potential in A549 cells. Combination chemotherapy with cisplatin- and etoposide-induced significant mitochondrial dysfunction by causing reduction in A549 cell $\Delta\Psi_m$, whereas pretreatment of these cells with nicotine completely blocked chemotherapy-induced reduction in $\Delta\Psi_m$. * $P < 0.05$ control versus cisplatin + etoposide; † $P < 0.05$ cisplatin + etoposide versus nicotine + cisplatin + etoposide, $n = 3$.

and Mitotracker green using methods previously described (18). As shown in Figure 3D, combination chemotherapy with cisplatin and etoposide induced significant reduction in A549 cell $\Delta\Psi_m$, whereas pretreatment of these cells with nicotine completely blocked chemotherapy-induced reduction in $\Delta\Psi_m$. Collectively, these data suggest that nicotine plays an important role in the prevention of chemotherapy-induced apoptosis by causing inhibition of chemotherapy-induced mitochondrial damage and stabilization of $\Delta\Psi_m$ in A549 cells.

Nicotine Prevents Chemotherapy-Induced Up-Regulation and Translocation of Bax to the Mitochondria

Migration of Bax to the mitochondria is an important event associated with apoptosis. Recent studies demonstrate that the migration and mitochondrial translocation of Bax in response to oxidative stress is associated with apoptosis (20). Using immunocytochemical staining with the mitochondrial marker, Mitotracker red, in this study, we examined the effects of nicotine on chemotherapy-induced Bax activation in A549 cells. As shown in Figure 4, exposure of A549 cells to cisplatin and etoposide caused induction, translocation, and colocalization of Bax to the mitochondria, whereas pretreatment of cells with nicotine blocked these effects, suggesting a possible role of proapoptotic protein Bax in regulating the mitochondria-mediated death pathway in these cells.

Modulation of a Functional Electron Transport via Mitochondrial Voltage-Dependent Anion Channels by Nicotine in Part Prevents Chemotherapy-Induced Apoptosis in A549 Cells

To determine whether functional electron transport in the mitochondria plays a role in mediating effects of nicotine, we used the mitochondrial anion channel inhibitor, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), which prevents mitochondrial superoxide production. DIDS protects cells by blocking the egress of ROS from the mitochondria through

voltage-dependent anion channels. The A549 cells were treated with 10 μ M DIDS followed by 1 μ M nicotine for 24 hours and then cisplatin (35 μ M) for 24 hours. Apoptosis was assessed by using DNA nucleosomal fragmentation ELISA, as described above. As shown in Figure 5A, DIDS, in part, blocked the antiapoptotic effect of nicotine against cisplatin-induced apoptosis, suggesting that modulation of functional electron transport via mitochondrial voltage-dependent anion channels by nicotine plays an important role in mediating the antiapoptotic effects of nicotine against chemotherapy-induced apoptosis in A549 cells. In addition, DIDS partially blocked cisplatin-induced apoptosis, suggesting that cisplatin-induced mitochondrial dysfunction, in part, causes apoptosis in these cells. Furthermore, we examined the effects of DIDS on combination chemotherapy-induced apoptosis, with and without the inhibition of the Akt pathway, by using LY294002. As shown in Figure 5B, cisplatin and etoposide together induced a sixfold increase in DNA fragmentation ELISA; pretreatment of these cells with DIDS caused a significant reduction in combination chemotherapy-induced apoptosis, and caused partial inhibition of the protective effects of nicotine against chemotherapy-induced apoptosis in these cells. Furthermore, addition of LY294002 to DIDS completely blocked the antiapoptotic effects of nicotine, suggesting that the MAPK-mediated mitochondrial death pathway and Akt pathway both play a critical role in mediating the effects of nicotine and chemotherapy in these cells.

Antiapoptotic Effects of Nicotine Are Mediated by the Mitochondrial Signaling Pathway

As shown above, nicotine, as well as chemotherapeutic agents, in part mediated their effects via mitochondrial signaling pathways in A549 cells. To differentiate the role of the mitochondrial pathway in mediating the apoptotic effects of chemotherapy versus antiapoptotic effects of nicotine, we examined the effects of nicotine against TNF- α -induced apoptosis in A549 cells. TNF- α induces apoptosis via activation of the death receptor pathway. The A549 cells were treated with nicotine (1 μ M) for 24 hours

Figure 4. Nicotine prevents chemotherapy-induced up-regulation and translocation of Bax to the mitochondria. Exposure of A549 cells to cisplatin and etoposide caused induction, translocation and colocalization Bax to the mitochondria, whereas pretreatment of the cells with nicotine blocked chemotherapy-induced translocation and mitochondrial localization of Bax in A549 cells.

Figure 5. (A and B) Modulation of a functional electron transport via mitochondrial voltage-dependent anion channels by nicotine, in part, prevents chemotherapy-induced apoptosis in A549 cells. (A) The mitochondrial anion channel inhibitor, DIDS, in part, blocked the antiapoptotic effect of nicotine against cisplatin-induced apoptosis as well as cisplatin-induced apoptosis, as assessed by DNA fragmentation ELISA in A549 cells. * $P < 0.05$ control (dotted bars) versus cisplatin (hatched bars) or DIDS + cisplatin; † $P < 0.05$ cisplatin versus nicotine + cisplatin (open bars); * $P < 0.05$ cisplatin versus DIDS + cisplatin. Solid bars, nicotine. (B) An addition of LY294002 along with DIDS completely blocked the apoptotic effects of chemotherapy in A549 cells. * $P < 0.05$ control (dotted bars) versus cisplatin + etoposide (hatched bars) or DIDS + cisplatin + etoposide; † $P < 0.05$ cisplatin + etoposide versus nicotine + cisplatin + etoposide (open bars) or DIDS + nicotine + cisplatin + etoposide; * $P < 0.05$ cisplatin + etoposide versus DIDS or DIDS + LY294002 + cisplatin + etoposide, $n = 3$. Solid bars, nicotine. (C and D) Antiapoptotic effects of nicotine are mediated by mitochondrial death pathway. (C) Nicotine prevents TNF- α -induced apoptosis in A549 cells, as assessed by DNA nucleosomal fragmentation

ELISA and TUNEL assays. * $P < 0.05$ control versus TNF- α ; † $P < 0.05$ TNF- α versus nicotine + TNF- α , $n = 3$. (D) Inhibition of a functional electron transport via mitochondrial voltage-dependent anion channels by DIDS blocks the protective effects of nicotine against TNF- α -induced apoptosis, whereas DIDS does not block TNF- α -induced apoptosis in A549 cells.

followed by TNF- α (100 ng). Apoptosis was assessed by using DNA fragmentation ELISA and terminal transferase dUTP-digoxigenin nick end-labeling assay. As shown in Figures 5C and 5D, TNF- α -induced apoptosis was completely prevented by nicotine in A549 cells. Furthermore, we examined the effects of inhibition of mitochondrial voltage-dependent anion channels by DIDS in mediating protective effects of nicotine against TNF- α -induced

apoptosis. DIDS inhibits functional electron transport in mitochondrial anion channels, thus preventing activation of the mitochondrial signaling pathway. The A549 cells were treated with DIDS, followed by nicotine (1 μ M) for 24 hours, and then TNF- α . As shown in Figure 5D, DIDS blocked the antiapoptotic effects of nicotine against TNF- α -induced apoptosis in A549 cells; however, DIDS did not prevent TNF- α -induced apoptosis,

suggesting a role of the mitochondrial signaling pathway in mediating antiapoptotic effects of nicotine in lung cancer cells.

Mitochondria Play a Crucial Role in Mediating the Antiapoptotic Effects of Nicotine, as well as in Chemotherapy-Induced Apoptosis in A549 Cells

We generated A549-p0 cells that lack mitochondrial DNA by slow chemical elimination of mitochondrial DNA, culturing cells in a medium supplemented with ethidium bromide (25 ng/ml), sodium pyruvate (1 mM), and uridine (50 μ g/ml), by the methods described by King and Attardi (16), which was also used in our previous studies (15–17). The lack of normal oxidative phosphorylation in these cells was confirmed by comparing antimycin-A-induced ROS formation in A549 and A549-p0 cells by using dichlorofluorescence assay, as previously described (15). The A549-p0 cells were treated with nicotine, followed by cisplatin, with or without etoposide, using similar study conditions used in our above experiments. As shown in Figures 6A–6C, A549-p0 cells showed a slightly higher amount of baseline apoptosis as compared with wild-type A549 cells. However, these mitochondria-deprived cells demonstrated relative resistance to chemotherapy-induced apoptosis, and showed a slight decrease in $\Delta\Psi_m$ as compared with wild-type A549 cells. Nicotine failed to show its antiapoptotic effect or to modulate chemotherapy-induced reduction in the $\Delta\Psi_m$ by A549-p0 cells. Moreover, pretreatment of A549-p0 cells with LY294002 did not show a change in chemotherapy-induced apoptosis in these cells. These data demonstrate that the presence of intact mitochondrial DNA is crucial in mediating the antiapoptotic effects of nicotine, as well as for causing chemotherapy-induced apoptosis in A549 cells. Furthermore, as shown in Figure 6D, nicotine caused phosphorylation of proapoptotic protein Bcl2-antagonist of cell death (Bad), suggesting that nicotine-induced phosphorylation of Bad via the Akt pathway may inhibit chemotherapy-induced apoptosis by preventing activation of the mitochondrial death pathway in A549 cells.

DISCUSSION

In this study, we demonstrated that nicotine inhibits chemotherapy-induced apoptosis by the modulation of the mitochondrial death pathway in lung cancer cells. Nicotine was noted to inhibit cisplatin- and etoposide-induced apoptosis in A549 cells. Nicotine prevented chemotherapy-induced reduction of mitochondrial membrane potential, activation of caspase-9, and translocation of Bax to the mitochondria. Akt mediated phosphorylation of proapoptotic protein Bad, and up-regulation of antiapoptotic protein, XIAP, was seen in cells exposed to nicotine. Furthermore, differential activation of MAPK was found to play a role in mediating chemotherapy-induced apoptosis, as well as antiapoptotic effects of nicotine in these cells. MEK1 mediated the antiapoptotic effects of nicotine, whereas MEK2 mediated chemotherapy-induced apoptosis. Regulation of the mitochondrial signaling pathway appeared to be critically involved in mediating the effects of nicotine as well as chemotherapy in lung cancer cells. Using the mitochondrial anion channel inhibitor, DIDS, we demonstrated that the antiapoptotic effects of nicotine against TNF- α -induced apoptosis are mediated by the mitochondrial signaling pathway in lung cancer cells. Furthermore, using A549-p0 cells that lack mitochondrial DNA and functional electron transport, we demonstrated that the intact mitochondria play a central role in mediating antiapoptotic effects of nicotine in these cells. The mitochondrial regulation of nicotine imposes an important mechanism that can critically impair the treatment of lung cancer, as activation of the mitochondrial death pathway is one

of the most common mechanisms by which many cancer-therapeutic agents, including drugs, radiation, and heat treatments, induce apoptosis in the tumor cells.

The biological effects of nicotine are mediated by nicotine acetylcholine receptors. High-affinity nAChRs are widely expressed in both human lung cancer cells and normal lung cells (8–10, 21). Dasgupta and colleagues and others (10, 14, 21) recently demonstrated that human non-small cell lung cancer cells (A549, H23, H441, and H1299) show abundant expression of $\alpha 3$ -, $\alpha 4$ -, $\alpha 5$ -, $\alpha 7$ -, and $\alpha 10$ -nAChRs subunits. The antiapoptotic effects of nicotine were observed in A549, H441, and H23 non-small cell lung cancer cells in our study. Multiple signaling pathways have been implicated in mediating effects of nicotine via nAChRs in various cells. Nicotine is known to activate intracellular Ca^{2+} release and modulate Raf/MEK/extracellular signal-regulated kinases, protein kinase C, Akt, NF- κ B, XIAP, Bcl2, and c-Myc-dependent cell survival and proliferation (21–25). Our findings corroborate these previously published data showing that MAPK and Akt pathways mediate antiapoptotic effects of nicotine in these cells. We also found that the differential regulation of MAPKs mediated the effects of nicotine and chemotherapy; MEK1 mediated the cell survival effects induced by nicotine, whereas MEK2 mediated chemotherapy-induced apoptosis. Nicotine induces Akt-mediated up-regulation of antiapoptotic protein, XIAP, which contributes to nicotine-mediated inhibition of apoptosis. Although, nicotine-induced activation of various kinases (MAPK, Akt, protein kinase C, etc.) via nAChRs are known to regulate downstream mitochondrial signaling, nicotine has also been reported to cause neuroprotective effects directly through the interactions with mitochondria, independent of the nAChRs (24, 25). Thus, intact mitochondria and mitochondrial signaling are critical in mediating antiapoptotic effects of nicotine, as shown in our study.

Several cancer-therapeutic agents, including drugs, radiation, and heat treatments, appear to kill tumor cells by inducing apoptosis by activation of the mitochondrial death pathway (6, 7). Therefore, nicotine modulation of mitochondrial signaling at multiple levels of the signaling cascade appears to be imperative in the prevention of chemotherapy-induced apoptosis in lung cancer. Oxidative stress induced by chemotherapy agents activates the intrinsic or mitochondrial death pathway, resulting in permeabilization and $\Psi\Delta m$, which follows the release of various proapoptotic mediators, such as cytochrome c, caspase-9, and apoptosis-inducing factor (24, 26). Our data demonstrate that nicotine prevents chemotherapy-induced reduction in mitochondrial membrane potential and blocks caspase-9 activation in A549 cells. The translocation and localization of proapoptotic proteins, Bax, Bak, and Bid, from the cytosol to the mitochondria, is required to induce cell death by activation of the mitochondrial apoptotic pathway (20, 24, 26). Consistent with these previous reports, we found that chemotherapy induces mitochondrial translocation of the proapoptotic protein, Bax; however, nicotine blocks these effects (20, 24). Nicotine induces Akt-mediated phosphorylation of proapoptotic protein, Bad, thus preventing downstream activation of the mitochondrial signaling pathway (14, 25). The mitochondrial electron transport chain generates ROS, which are then transported into the cytoplasm through voltage-dependent anion channels. The mitochondrial anion channel inhibitor, DIDS, protects cells from oxidative injury by blocking the egress of mitochondrial ROS into the cytoplasm (27). Inhibition of superoxide release by DIDS from the mitochondrial matrix prevents mitochondrial damage by blocking the $\Psi\Delta m$ and depolarization of the mitochondria. Cormier and colleagues and others (24, 28)

Figure 6. Mitochondria play a crucial role in mediating the antiapoptotic effects of nicotine as well as in chemotherapy-induced apoptosis in A549 cells. (A) A549-p0 cells showed slightly higher baseline apoptosis; however, they show resistance to cisplatin-induced apoptosis as compared with wild-type A549 cells, whereas nicotine failed to show any antiapoptotic effect. * $P < 0.05$ control versus cisplatin; † $P < 0.05$ cisplatin (hatched bars) versus nicotine + cisplatin (open bars); ‡ $P < 0.05$ cisplatin in A549 cells versus cisplatin in A549-p0 cells, $n = 3$. Solid bars, nicotine; dotted bars, control. (B) Pretreatment of A549-p0 cells with LY294002 blocked the apoptotic effects of chemotherapy and the effects of nicotine in A549-p0 cells. * $P < 0.05$ control (dotted bars) versus cisplatin + etoposide (hatched bars) in A549 cells or A549-p0 cells, or LY294002 in A549-p0 cells; † $P < 0.05$ cisplatin + etoposide versus nicotine + cisplatin + etoposide (open bars), ‡ $P < 0.05$ cisplatin + etoposide in A549 cells versus cisplatin + etoposide in A549-p0 cells, $n = 3$. Solid bars, nicotine. (C) A549-p0 cells showed a modest decrease in $\Delta\Psi_m$ on exposure to cisplatin; however, nicotine did not modulate $\Delta\Psi_m$ induced by cisplatin + etoposide in A549-p0 cells. * $P < 0.05$ control versus cisplatin + etoposide in A549-p0 cells, $n = 3$. (D) Nicotine induces phosphorylation of proapoptotic protein Bad in A549 cells, as assessed by Western blot assay.

recently reported that nicotine significantly decreases superoxide anion generation in brain mitochondria by causing a direct effect of nicotine on the mitochondria respiratory chain, independent of its receptor. In our study, we found that DIDS blocked the antiapoptotic effects of nicotine, whereas it

did not modulate TNF- α -induced apoptosis, suggesting the role of mitochondrial signaling in mediating antiapoptotic effect of nicotine in lung cancer cells. In addition, antiapoptotic effects of nicotine, in part, were mediated by Akt signaling pathway in these cells.

Figure 7. Schematic diagram of nicotine-mediated mitochondrial signaling in the prevention of chemotherapy-induced apoptosis in lung cancer. ERK = extracellular signal-regulated kinase.

Furthermore, we found that intact mitochondria and mitochondrial function are critical in mediating antiapoptotic effects of nicotine as well as chemotherapy-induced apoptosis in A549 cells. To determine the role of mitochondria, we generated A549-p0 cells that lack mitochondrial DNA by the ethidium bromide technique (15–17). These cells showed substantial decreases in ROS production. The lack of normal oxidative phosphorylation in these cells was confirmed by comparing antimycin-A-induced ROS formation in A549 and A549-p0 cells by using dichlorofluorescein assay (15). The mitochondria-deprived cells demonstrated a relative resistance to cisplatin-induced apoptosis as compared with wild-type A549 cells, and showed a modest decrease in $\Delta\Psi_m$ on exposure to cisplatin, whereas nicotine failed to show any antiapoptotic effects or modulate the $\Delta\Psi_m$ induced by cisplatin in A549-p0 cells. Moreover, addition of Akt inhibitor did not alter these results.

These data suggest that intact mitochondria play a critical role in mediating antiapoptotic effects of nicotine as well as chemotherapy-induced apoptosis in lung cancer cells. A hypothetical model based on our results, showing the mechanisms underlying antiapoptotic effects of nicotine via modulation of mitochondrial signaling in A549 cells, is shown in Figure 7.

In summary, we show that nicotine prevents chemotherapy-induced apoptosis in human lung cancer cells by modulating the mitochondrial signaling pathway. These effects of nicotine are critically important, especially in patients undergoing treatment of lung cancer, as activation of the mitochondrial death pathway is one of the most common mechanisms by which many cancer-therapeutic agents induce apoptosis in tumor cells. Our findings are consistent with clinical studies showing that patients who continue to smoke have worse survival, possibly due to tumor progression and resistance to cancer therapy (2). We speculate that active smoking, as well as nicotine supplementation, may reduce the response to chemotherapeutic agents. Strategies aimed at understanding nicotine-mediated signaling may facilitate the development of improved therapies for lung cancer.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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