Tobacco components stimulate Akt-dependent proliferation and NFκB-dependent survival in lung cancer cells

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Retrospective studies have shown that patients with tobacco-related cancers who continue to smoke after their diagnoses have lower response rates and shorter median survival compared with patients who stop smoking. To provide insight into the biologic basis for these clinical observations, we tested whether two tobacco components, nicotine or the tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), could activate the Akt pathway and increase lung canc
er cell proliferation and survival. Nicotine or NNK, rapidly and potently, activated Akt in non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC) cells. Nicotinic activation of Akt increased phosphorylation of multiple downstream substrates of Akt in a time-dependent manner, including GSK-3, FKHR, tuberin, mTOR and S6K1. Since nicotine or NNK bind to cell surface nicotinic acetylcholine receptors (nAchR), we used RT-PCR to assess expression of nine alpha and three beta nAchR subunits in five NSCLC cell lines and two types of primary lung epithelial cells. NSCLC cells express multiple nAchR subunits in a cell line-specific manner. Agonists of α3/α4 or α7 subunits activated Akt in a time-dependent manner, suggesting that tobacco components utilize these subunits to activate Akt. Cellular outcomes after nicotine or NNK administration were also assessed. Nicotine or NNK increased proliferation of NSCLC cells in an Akt-dependent manner that was closely linked with changes in cyclin D1 expression. Despite similar induction of proliferation, only nicotine decreased apoptosis caused by serum deprivation and/or chemotherapy. Protection conferred by nicotine was NFκB-dependent. Collectively, these results identify tobacco component-induced, Akt-dependent proliferation and NFκB-dependent survival as cellular processes that could underlie the detrimental effects of smoking in cancer patients.

Abbreviations: DMXB, 3-[2,4-dimethoxybenzylidene] anabaseine; DnAkt, dominant negative Akt; GSK, glycogen synthesis kinase; HA, hemagglutinin; MDM2, mouse double minute 2 homologue; mTOR, mammalian target of rapamycin; nAchR, nicotinic acetylcholine receptors; NFκB, nuclear factor kappa B; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NSCLC, non-small cell lung cancer; PI3K, phosphotidylinositol 3-kinase; PARP, poly (ADP ribose) polymerase; SCLC, small cell lung cancer.

Introduction

Lung cancer is the leading cause of cancer death throughout the world, causing ~1.2 million deaths annually (1). The development of lung cancer is associated with smoking in ~85–90% of cases (2). The basis for marked lethality is probably on account of the high frequency of advanced stage at diagnosis and the inherent therapeutic resistance of lung cancer cells. Smokers continue to have additional health risks, even after the diagnosis of a tobacco-related cancer. These risks include the increased risk of developing a second tobacco-related malignancy, possibly because of field carcinogenesis effects (3,4). In addition to the prospective risks, retrospective studies have shown that continued smoking during therapy for tobacco-related cancers, such as lung cancer is associated with lower response rates to chemotherapy and/or radiation, and in some cases, decreased survival (5–7). Thus, the identification of molecular events associated with exposure to tobacco components that contribute to therapeutic resistance might provide a basis for interventions aimed at ameliorating the effects of smoking during therapy for the patients unable to quit smoking.

Although changes in oncogenes or tumor suppressor genes are related to smoking and could contribute to therapeutic resistance (8), activation of signal transduction pathways that promote cellular survival such as the phosphotidylinositol 3-kinase (PI3K)/Akt pathway might also contribute to tobacco-related therapeutic resistance. The PI3K/Akt pathway is a critical pathway in cancer because it contributes to tumorigenesis, tumor growth and therapeutic resistance [reviewed in (9)]. Akt serves at a nodal point in this pathway and is probably important for the development and maintenance of lung cancer. Active Akt has been detected in human lung cancer precursor lesions and in established lung cancers (10). Non-small cell lung cancer (NSCLC) cells frequently have constitutively active Akt that promotes cellular survival and resistance to chemotherapy or radiation (11). In primary human lung epithelial cells, tobacco components such as nicotine or the tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), activate the Akt pathway which increases cellular proliferation and confers protection against different cellular stresses (12). Moreover, tobacco carcinogen-induced transformation of bronchial epithelial cells increases activation of the Akt pathway in vitro and in vivo (13). These studies suggest that Akt activation is an early event that is related to exposure to tobacco components, but no study has explored the effects of this mechanism of Akt activation in established lung cancer cells. This is highly relevant because smokers diagnosed with tobacco-related cancers are often not encouraged to quit smoking because of a perception that it is ‘too late’.

Here, we report the rapid and potent activation of Akt by tobacco components in lung cancer cells, and describe changes in cellular proliferation and survival that occur after administration of these components. These studies identify signaling mechanisms and cellular processes that could contribute to the
poor outcomes associated with patients who smoke during cancer therapy.

**Materials and methods**

**Materials**

Etoposide and paclitaxel were purchased from Calbiochem (La Jolla, CA). All phospho-specific antibodies and poly (ADP ribose) polymerase (PARP) antibodies were from Cell Signaling Technology (Beverly, MA). α-7 nicotinic acetylcholine receptors (nAChR) and α-tubulin antibodies were from Sigma-Aldrich (St Louis, MO). α4 nAChR, β2 nAChR, cyclin D1 antibodies and hemagglutinin (HA)-probed F7 were from Santa Cruz Biotechnology (Santa Cruz, CA). Protease inhibitor cocktail was obtained from Sigma Chemical, and protein assay materials were from Bio-Rad (Hercules, CA). All cell culture reagents were purchased from Life Technologies (Rockville, MD). Protran Pure nitrocellulose membranes were purchased from Schleicher & Schuell (Dassel, Germany). Nicotine was from Sigma-Aldrich. NNK was from ChemSyn Laboratories (Lenexa, KS). LY294002 and (Dassel, Germany). Nicotine was from Sigma-Aldrich. NNK was from ChemSyn Laboratories (Lenexa, KS). LY294002 and α-anotoxan (ATX) were purchased from Sigma-Aldrich. 3-(2,4)-Dimethoxybenzylidine anabaseine (DMXB) was a generous gift from Dr William Kem, University of Florida.

**Cell culture**

All lung cancer cell lines were established at the National Cancer Institute/Naval Medical Oncology (Bethesda, MD) or were purchased from ATCC (Manassas, VA). H157V and H157I cells were a generous gift from Dr David Jones, University of Virginia. H157 and H1703 cells were maintained in 75 cm² flasks in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. H69, H157V and H157I cells were maintained in RPMI 1640 supplemented with 10% (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were incubated at 37°C in a 7.0% CO₂ atmosphere incubator. The stock cultures were split on a weekly basis at 1:5 or 1:10 ratio.

**Pharmacological treatments**

For time-dependent induction of Akt phosphorylation or assessing phosphorylation of downstream substrates, cells were serum deprived in 0.1% FBS containing media for 24 h prior to addition of 10 µM nicotine or 100 nM NNK. Dose-response curves were generated at 60 min. α-ATX (20 µM) or DMXB (20 µM) was added to H157 cells after serum deprivation. For examination of cell proliferation, serum-deprived H157 or H1703 cells were treated daily with nicotine or NNK. To assess apoptosis, H157 and H1703 cells were treated with paclitaxel (100 nM) or etoposide (100 µM) for 48 h in 0.1% FBS in the absence or presence of nicotine (10 µM) or NNK (100 nM). For apoptosis of H157V or H157I cells, cells were plated in media containing 10% FBS. After attachment, cells were washed twice with PBS and incubated in 0.1% FBS-containing medium for 48 h in the absence or presence of nicotine or NNK.

**Adenoviral infection**

Adenoviruses containing β-galactosidase (β-gal) or dominant negative Akt (DnAkt) were generous gifts from Dr Kenneth Walsh and have been described previously (14). Cells were plated at a density of 3 × 10⁵ cells per well in 12-well plates. After attachment, cells were washed with PBS, and infected with media containing adenoviral constructs for β-gal or DnAkt [multiplicity of infection (MOI) 50] for 24 h. Infected cells were then treated with or without nicotine or NNK and cells were harvested and analyzed for cell proliferation as described below. Parallel samples were harvested for immunoblotting and quantification of cell number in each experiment.

**Immunoblotting**

After the various pharmacologic treatments described above, cell extracts were prepared as described previously (11). Protein yield was quantified using the Bio-Rad DC Protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equivalent protein was loaded, and the lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. Equivalent loading was confirmed by staining of membranes with fast green. Membranes were blocked for 1 h in blocking buffer [1 x Tris buffered saline (TBS), 5% milk and 0.2% Tween-20] and placed in primary antibody (1 x TBS, 5% milk, 0.1% Tween-20 and 1:1000 antibody) overnight at 4°C. Membranes were washed three times in washing buffer [0.1% NP40, 0.1% Tween-20 and 1 x TBS]. Primary antibody was detected using horseradish peroxidase-linked goat anti-mouse or goat anti-rabbit IgG antibodies and visualized with the enhanced chemiluminescent detection system ECL (Amersham Pharmacia Biotech, Amersham). All phospho-specific antibodies were used at 1:1000 dilutions. Immunoblot experiments were performed at least three times.

**Cell proliferation assays**

Cells were seeded in triplicate in 12-well plates at a density of 3 × 10⁴ cells per well and incubated overnight. Cells were then washed twice with PBS and media were changed to serum-free medium. Where indicated, nicotine or NNK was added daily. Cells were harvested at 72 h by trypsinization and either counted with a ZEiss Coulter Counter (Beckman Coulter, Miami, FL), or fixed in ice-cold 70% methanol and stained with crystal violet and the absorbance at 540 nm in each sample was measured using EL x 800 Universal Microplate Reader (BIO-TEK Instruments, VT). Proliferation experiments were performed three times.

**Apoptosis assays**

Floating and adherent cells were harvested by trypsinization and then centrifuged at 1000 × g for 5 min. Cells were fixed in ice-cold 70% methanol added dropwise and then incubated at −20°C for 30 min. Cells were centrifuged and incubated with propidium iodide (25 µg/mL) supplemented with RNaseA for 30 min at room temperature. Quantification of sub-2N DNA was determined by flow cytometry analysis using a Beckton Dickinson FACSort and by manual gating using CellQuest software. Gating was performed on blinded samples. All apoptotic experiments were repeated three times.

**Reverse transcription-PCR**

Total RNA extraction and PCR reaction mixtures were as described previously (11). Subunit specific primers for nAChR were synthesized by Sigma-Genosys, TX, with the following sequences: α1: 5'-CGTCTGTTGGAAGATC-3' (sense), 5'-CCGGCTCTCATGAATT-3' (antisense); α2: 5'-CGGTTGAC-TCTGTAG-3' (sense), 5'-CAGATCTTACGCAGT-3' (antisense) (15); α3: 5'-CATGTCATCAGCAGT-3' (sense), 5'-GTCCTGAGGCTC-3' (antisense) (16); α4: 5'-CTCTGACACCACACCT-3' (sense), 5'-AGACCGTCTCCGCTCC-3' (antisense) (17); α5: 5'-TCATGTJAGACAGTACTTT-3' (sense), 5'-ATTGCCCCATTTATATAA-3' (antisense) (18); α6: 5'-GGCTTCGAGAAAAGC-3' (sense), 5'-AAGATTTCCTGTTTCC-3' (antisense) (19); α7: 5'-CAACTGTCGGCTACACCCGATGTC-3' (sense), 5'-CTCAGTGCCCTCGTGGATCGTGA-3' (antisense) (20); α8: 5'-GTCGACGGTCTTGGT-3' (sense), 5'-ATTCGCCCTTGTATGAT-3' (antisense) (21); α9: 5'-CTGTTCCGGTGACCTTTT-3' (sense), 5'-GAAAGGCTGCTACATCC-3' (antisense) (22); β2: 5'-CAACTCTACATGGTC-3' (sense), 5'-GTCCGTTGCTGATCC-3' (antisense) (23); β3: 5'-AGAGGCTCTTTCGTCAGA-3' (sense), 5'-GCCACATCTTCAAGACAG-3' (antisense) (24); β4: 5'-CTGAAACAGGGAAGCT-3' (sense), 5'-CCATGTACTTCCGGTGT-3' (antisense) (15); and β-actin primers were 5'-GTACCGGGTCCCAGCACA-3' (sense) and 5'-CTCTTAACTGTCACACAGTTT-3' (antisense). α9 and α10 DNA controls were generously provided by Dr L. Lustig, Johns Hopkins University. nAChR primers yielded predicted products of 505 (α1), 466 (α2), 401 (α3), 371 (α4), 265 (α5), 413 (α6), 598 (α7), 403 (α9), 388 (α10), 347 (β2), 354 (β3) and 310 (β4) bp.

**Luciferase assays**

H157V and H157I cells were plated at 3 x 10⁴ cells per well in 24-well plates. Cells were transiently transfected the next day with 1 µg DNA PathDetect NFkB cis-reporting system vector (Strategene, La Jolla, CA) using Superfect transfection reagent (Qiagen, Valencia, CA). According to manufacturer’s instructions. After incubation for 24 h, media were changed to fresh RPMI containing 10% FBS or 0.1% FBS with or without nicotine (100 nM). Cells were lysed 24 h later with 1 x Reporter Lysis Buffer (Promega, Madison, WI) and stored frozen at −20°C overnight. Luciferase activity was determined by testing 20 µl of cell lysate and 100 µl of Luciferase Assay Reagent (Promega) using a moonlight 2010 tube luminometer (Analytical Luminescence Laboratory, San Diego, CA). Relative luciferase units were normalized to total protein levels from the cell lysates. Luciferase assays were performed three times.

**Statistical analysis**

Statistical comparison of mean values was performed using the Student t-test. All P values are two tailed.

**Results**

Nicotine or NNK increases activation of Akt in a dose-dependent and time-dependent manner

To investigate whether nicotine or NNK could stimulate the Akt pathway in fully transformed lung cancer cells, we used two NSCLC cell lines (H157 and H1703) and one small cell lung cancer (SCLC) cell line (H69) to assess phosphorylation of Akt at S473, which is indicative of Akt activation. Nicotine

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Fig. 1. Time- and dose-dependent induction of Akt activation by nicotine or NNK. (A) Nicotine. Lung cancer cell lines were plated on 6-well plates in DMEM with 10% FBS overnight. Media were then changed to DMEM with 0.1% FBS, with or without addition of nicotine (10 μM) for indicated times. Cells were harvested and processed for immunoblotting using phospho-specific antibodies against S473 and antibodies against total Akt. Scanned images of immunoblots were analyzed using NIH Image 1.62. Each pAkt signal was normalized to native Akt, and the bars indicate the fold induction of the pAkt signals compared with the control. (B) NNK. Cell extracts were prepared as in (A), except that NNK (100 nM) was administered for the indicated times. (C) Dose dependence. Cells were plated, treated, and processed as in (A), except that cells were harvested at 45 min.
or NNK rapidly increased Akt activation in all three cell lines (Figure 1A and B). Nicotine increased Akt activation 2- to 3-fold within 15–30 min (Figure 1A). Similar induction of Akt activation was observed with NNK, with 2- to 3-fold induction observed by 30 min (Figure 1B). Nicotine or NNK did not affect total levels of Akt expression. In experiments designed to test dose-dependent responses to nicotine, nicotine increased Akt phosphorylation in H157 cells with doses as low as 10 nM, but maximum Akt phosphorylation in H157 or H1703 cells was observed at 1–10 μM (Figure 1C). These concentrations might be achievable in smokers, because average steady-state serum concentrations of nicotine have been reported at ~200 nM, and acute increases to 10–100 μM in serum or to 1 mM at the mucosal surface immediately after smoking have been reported (20–22). NNK was more potent than nicotine, because increased phosphorylation of Akt was observed with doses as low as 1 nM in both cell lines. These studies demonstrate that nicotine, the addictive component of tobacco, and NNK, an important tobacco-specific carcinogen, activate Akt within minutes at doses that could be physiologically relevant.

**Nicotine or NNK increases phosphorylation of downstream substrates of Akt**

To demonstrate that activation of Akt by nicotine or NNK propagated signaling cascades within lung cancer cells, we initially assessed phosphorylation of downstream substrates using an antibody raised against the consensus sequence identified in all known Akt substrates (RXRXXS/T). Nicotine or NNK induced a time-dependent increase in phosphorylation of numerous downstream substrates in H157 (Figure 2A) and H1703 cells (Figure 2B) that reflected the rapid onset of increased Akt phosphorylation. The pattern of induction for H157 cells was different from H1703 cells, possibly reflecting differences between these cell lines in the status of molecules that control the Akt pathway such as PTEN or K-Ras (11). To identify which known Akt substrates were altered by nicotine or NNK treatment and to rule out contribution of kinases such as SGK that have similar consensus sequences to that of Akt, H157 cell extracts were probed with phospho-specific antibodies against glycogen synthase kinase (GSK-3), mouse double minute 2 homologue (MDM2), ASK1, FKHR, tuberin, and mammalian target of rapamycin (mTOR), which are direct Akt substrates, and eukaryotic initiating factor 4 binding protein 1 (4EBP-1) and S6 kinase 1 (S6K1), which are not direct Akt substrates but are phosphorylated in response to Akt activation (Figure 2C). Nicotine increased phosphorylation of all proteins tested within minutes (Figure 2C, left panels), although the time course varied in a substrate-specific manner. The greatest induction of phosphorylation was observed in substrates that control transcription and protein translation. NNK induced similar effects on phosphorylation of these proteins (Figure 2C, right panels), except that NNK did not induce MDM2 or mTOR phosphorylation. Variability in phosphorylation of Akt substrates could reflect differential sensitivity to regulatory mechanisms such as cellular phosphatases or the contribution of other kinases. Collectively, these data show that nicotine- or NNK-mediated stimulation of Akt results in broad propagation of the pathway, but that nicotine or NNK propagates the Akt signal differently.

**Identification of nAchR in lung cancer cells**

Nicotine or NNK exerts its biological effects through binding to nAchR. nAchR were originally described and are predominantly expressed in neural tissue, but nAchR have recently been reported to be expressed in other cell types, including small cell lung cancer (SCLC) cells (23–25). Functional nAchR are composed of homopentamers of α7-α10 subunits or heteropentamers derived from 5 α (α2–α6) and 3 β (B2–B4) subunits. α3- or α4-containing nAchR are most abundant in neural tissue (26), and α7-containing nAchR have been described in normal human bronchial epithelial and endothelial cells (16). To evaluate expression of individual nAchR subunits in NSCLC cell lines and to compare

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**Fig. 1. Continued.**

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expression with normal human airway epithelial cells, we performed nAchR subunit-specific RT-PCR analysis of α1-α10 and β2-β4 subunits (Table I). In the five NSCLC cell lines, α4, α5, α7 and β2 subunits were ubiquitously expressed. α1, α2, α10 and β3 subunits were not expressed in any NSCLC cell line tested, and α3, α6, α9 and β4 subunits were expressed in an NSCLC cell line specific manner. Differences in nAchR subunit expression were also observed.

Fig. 2. Time-dependent induction of Akt substrate phosphorylation by nicotine or NNK. (A) H157 cells. Cells were plated on 6-well plates and incubated in DMEM with 10% FBS overnight. Media were then changed to 0.1% FBS and cells treated with nicotine or NNK for the indicated time periods. Immunoblotting was performed with phospho-specific antibodies that recognize phosphorylated Akt substrates (RXRXXS/T) (panels A and B). Equivalent loading is shown by immunoblotting for α-tubulin. Extracts of NIH3T3 cells were included as controls. Asterisks indicate bands whose intensity increased or decreased after exposure to tobacco components. (B) H1703 cells. Same as (A) except that H1703 cells were used. (C) Effects of nicotine or NNK on phosphorylation of individual Akt substrates. H157 cells were treated similarly with nicotine or NNK for immunoblotting experiments performed with phospho-specific antibodies directed against individual downstream substrates at the indicated sites or with other antibodies. Immunoblots from nicotine- (left panels) or NNK-treated (right panels) cells are shown for a representative experiment done three times. Direct Akt substrates are shown by labels shaded in gray. Equivalent loading was confirmed by staining membranes with fast green.
between normal airway epithelial cells and NSCLC cells, such as loss of α10 subunits in all five NSCLC cell lines, but the significance of these differences is unknown. To confirm the results of the RT–PCR analysis, we performed immunoblotting with antibodies against α4, α7 and β2 nAchR subunits (Figure 3A). Each of these subunits was expressed at a protein level in H157, H1703 and A549 cells. These studies suggest that functional α4β2 and α7 nAchR could be expressed in NSCLC cells.

To identify nAchR that might be capable of activating Akt in NSCLC cells, we tested if subunit-specific nicotinic agonists could stimulate Akt activation (Figure 3B). α3/α4 agonist (27), increased Akt activation in H157 and H1703 cells within minutes. The onset of α3/α4 agonist-induced Akt activation was slower in H157 cells than in H1703 cells, where Akt activation increased ~3-fold within 5 min (densitometry not shown). DMXB, an α7 agonist (28), also increased Akt activation within minutes, and this response was prolonged in the H157 cells. Together, these data show that α3/α4 or α7 nAchR agonists can activate Akt in NSCLC cells.

Nicotine and NNK are Akt-dependent mitogens for NSCLC cells

Activation of the PI3K/Akt/mTOR pathway promotes cellular proliferation in multiple cell types. To determine if nicotinic activation of Akt induced NSCLC cell proliferation, different doses of nicotine were added daily to H157 cells in 0.1% FBS and cell number was determined after 5 days (Figure 4A). At doses as low as 100 nM, nicotine increased proliferation 2- to 4-fold. With higher doses, increased proliferation was observed compared with sham-treated cells, but dose-dependent increases in proliferation were not consistently

Table I. RT–PCR expression of nAchR subunits

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Total RNA was isolated from primary human airway epithelial cells (NHBE, normal human bronchial epithelial cells; SAEC, small airway epithelial cells) or NSCLC cell lines as described, and RT–PCR was performed to analyze individual nAchR subunits.
observed, perhaps due to the cumulative toxicity of daily dosing of 1 or 10 μM nicotine. Increased proliferation of H157 cells after nicotine treatment (10 μM) was associated with a 3.5-fold increase in Akt activation and increased cyclin D1 levels (Figure 4A, right panel).

To extend these studies and minimize the contribution of cumulative toxicity, the ability of lower doses of daily nicotine or NNK to induce proliferation was compared in H157 or H1703 cells after 3 days of exposure (Figure 4B). Nicotine or NNK (100 nM) increased proliferation ~2-fold in both cell lines. Immunoblotting analysis revealed that after daily administration of nicotine or NNK, Akt activation increased 4.15-fold in both cell lines. The reasons for the less robust increase in Akt activation on day 3 is unclear, but might be related to the higher dose (10 μM) or longer time course (5 days) used in Figure 4A. Increased NSCLC cell proliferation was associated with increased cyclin D1 expression, but was not accompanied by decreased levels of the cdk inhibitor, p27 (data not shown). (Expression of the cdk inhibitor p21 was not detectable in these cells.) These studies show that nicotine or NNK are mitogenic for NSCLC cells, which was associated with increased expression of cyclin D1.

To determine the relative contribution of Akt activation to tobacco component-mediated NSCLC cell proliferation, we used adenoviruses expressing mutant Akt (Figure 4C). H157 cells were infected with adenoviruses expressing β-gal or DnAkt, and nicotine- or NNK-induced NSCLC cell proliferation was assessed on day 3. When nicotine or NNK was added to cells infected with adenoviruses expressing β-gal, proliferation was increased ~2-fold. In contrast, when nicotine or NNK was added to cells expressing DnAkt, no increase in proliferation was observed. The inhibition of nicotine- or NNK-induced proliferation by mutant Akt was statistically significant (P < 0.05). In cells that were infected with DnAkt, expression of the HA epitope tag did not change with nicotine or NNK administration (top right panel). Inhibition of nicotine-induced proliferation correlated with an inhibition of cyclin D1 induction by nicotine or NNK, and was not due to an increase in apoptosis because nicotine or NNK did not induce PARP cleavage (Figure 4C, right panel). Thus, tobacco component-induced proliferation of NSCLC cells is dependent upon Akt activation.

**Nicotine increases NSCLC cellular survival and activation of NFκB**

To determine if nicotine or NNK could affect responsiveness to chemotherapy, NSCLC cells were treated with chemotherapeutic agents commonly used in the treatment of NSCLC, in the absence or the presence of nicotine or NNK (Figure 5A). Nicotine decreased paclitaxel-induced apoptosis in H157 cells by 40% (upper left panel), but NNK did not inhibit paclitaxel-induced apoptosis (upper right panel), even when higher doses were used (data not shown). Similar effects were observed in the H1703 cells, where nicotine decreased etoposide-induced apoptosis by 75% (lower left panel). NNK was again ineffective (lower right panel). To determine the contribution of the
Fig. 4. Effects of nicotine or NNK on NSCLC cell proliferation. (A) H157 cells were plated on 6-well plates in triplicate. Nicotine was added daily to the existing DMEM with 0.1% FBS. On day 5, the cells were harvested and counted with Beckman Coulter Counter or immunoblotting was performed for phospho-Akt, total Akt and cyclin D1 (right panels). Tubulin was used as a loading control. Asterisks indicate statistically significant differences between nicotine treatment and no treatment ($P < 0.05$). The fold increase in pAkt: total Akt is shown in the right lower panel. (B) H157 or H1703 cells were treated as in (A) except that lower doses of nicotine and NNK were used (100 nM), and the experiment was conducted for 3 days. Immunoblotting for phospho-Akt, total Akt and cyclin D1 is shown. Asterisks indicate statistically significant differences between treatment with tobacco components and no treatment ($P < 0.05$). The fold increases in pAkt: total Akt are shown in graphs below the corresponding immunoblots. (C) H157 cells were infected with adenoviruses expressing β-gal or DnAkt. After recovery for 24 h, cells were serum-deprived and nicotine or NNK was added as indicated for 3 days. Cells were harvested for assessment of proliferation using crystal violet staining and for immunoblotting for HA, cyclin D1 and PARP. Tubulin is shown as a loading control. Asterisks indicate statistically significant differences in proliferation between cells infected with β-gal or DnAkt and treated with nicotine or NNK ($P < 0.05$).
PI3K/Akt pathway to nicotine-mediated protection of H157 cells, we used the PI3K inhibitor, LY294002, in the absence or presence of nicotine and/or paclitaxel. As shown in Figure 5B, combining LY294002 with paclitaxel increased apoptosis above that observed with paclitaxel alone, consistent with prior studies from our laboratory (11). Although nicotine protected the cells from paclitaxel alone, the protective effect of nicotine was lost in the presence of LY294002, suggesting that the anti-apoptotic effects of nicotine are dependent upon the activation of the PI3K/Akt pathway.

Chemotherapeutic resistance can be associated with increased NFκB activity (29). To determine if nicotine-induced cellular survival was dependent upon NFκB activity, we utilized H157 cells that stably overexpress IκBa, which inhibits NFκB (H157I cells), or H157 cells that stably overexpress vector alone (H157V cells) (30). H157I cells have decreased NFκB activity and increased sensitivity to chemotherapy. Relative expression of IκBa in H157V or H157I cells is shown in Figure 6A (insets, upper right). When these cells were grown in the presence of 10% FBS and apoptosis was measured after 2 days, there was no change in basal levels of apoptosis in H157V or H157I cells, in the absence or presence of nicotine (left columns, Figure 6A). When these stably transfected cells were serum deprived, however, levels of apoptosis in H157I cells increased ~2.5-fold above that observed in H157V cells. Nicotine inhibited serum deprivation-induced apoptosis by 48% in H157V cells. The protection conferred by nicotine in H157V cells was not observed in H157I cells. To address whether the high levels of apoptosis in H157I cells caused by serum starvation precluded nicotine from protecting these cells from apoptosis, we performed a time course experiment with serum starved H157V and I cells with or without nicotine over 24, 48 and 72 h (data not shown). Basal levels of apoptosis increased progressively with time, but regardless of levels of apoptosis over 50% in H157V cells, nicotine was still able to protect these cells, indicating that the high levels of serum starvation-induced apoptosis do not preclude protection by nicotine. Interestingly, Akt activation was induced by ~1.5-fold in both H157I and H157V cells, indicating that pathways leading to Akt activation are intact in both cell types, suggesting that the ability of nicotine to promote cell survival is dependent on differences in signal propagation downstream of Akt (Figure 6A, insets, lower right). These experiments were repeated using NNK. Similar to the results obtained with parental H157 cells treated with chemotherapy, NNK did not protect H157V or H157I cells from serum deprivation-induced apoptosis (data not shown). These experiments showed that nicotine promoted survival of serum deprived NSCLC cells in an NFκB-dependent manner.

To show that nicotine directly stimulates NFκB activity, we transiently transfected H157V or H157I cells with an NFκB reporter and measured luciferase activity without or with exposure to nicotine (Figure 6B). When cells were grown in media with 10% FBS without nicotine, H157I cells had 65% less basal luciferase activity than H157V cells. In H157V cells grown in 10% FBS or 0.1% FBS, nicotine increased luciferase activity 2- to 3-fold. H157I cells did not increase luciferase activity in response to nicotine in either growth condition.
Thus, nicotine stimulates NFκB activity in NSCLC cells. Collectively, these studies show that nicotine is a survival factor for NSCLC cells under conditions of exposure to chemotherapy or serum deprivation, and that nicotine-mediated survival is dependent upon induction of NFκB activity.

**Discussion**

The activation of Akt and the promotion of lung cancer cell proliferation and survival by tobacco components recapitulates biologic processes that may underlie the poor clinical
outcomes for patients with tobacco-related cancers who continue to smoke. Moreover, these studies advance prior studies of nicotine and NNK by identifying Akt and NFκB as the key cellular targets of these tobacco components in established lung cancer cells. Activation of Akt by tobacco components has been observed in many epithelial cell systems relevant to lung cancer. The dose- and time-dependent activation of Akt by nicotine or NNK in lung cancer cells is similar to that we observed in primary cultures of human bronchial or small airway epithelial cells (12). Likewise,

Fig. 6. Role of NFκB in nicotine-mediated survival of NSCLC cells. (A) H157 cells overexpressing IκBα are resistant to nicotine protection. H157V or H157I cells were grown in media containing 10% FBS or 0.1% FBS, in the absence or presence of daily nicotine (10 μM) for 48 h, and apoptosis was assessed. Expression of IκBα, phosphorylated Akt and total Akt are shown. Tubulin and total levels of Akt are used as loading controls. Asterisks indicate statistically significant differences in apoptosis between addition of nicotine or no treatment (P < 0.05). (B) Nicotine activates NFκB activity in H157V but not H157I cells. H157V or H157I cells were transiently transfected with a luciferase reporter containing an NFκB promoter. Cells were grown in 10% FBS or 0.1% FBS for 48 h in the absence or presence of nicotine (10 μM) and luciferase activity was measured. Asterisks indicate statistically significant differences in apoptosis between addition of nicotine or no treatment (P < 0.05).
activation of Akt by nicotine or NNK has also been observed with human lung epithelial cells at intermediate steps in the transformation process (data not shown). Taken together, human lung epithelial cells throughout the phenotypic spectrum activate Akt in response to tobacco components in vitro. Inhibiting the induction of Akt activity by tobacco components might therefore be a valuable approach to mitigate the effects of smoking in smokers at risk for the development of lung cancer, and/or in lung cancer patients who continue to smoke.

Such approaches would need to consider the consequences of inhibiting nicotinic induction of Akt in other cell types, especially in neuronal tissues. In neuronal tissues, activation of Akt by tobacco components might ironically confer health benefits, which could hypothetically complicate interventions suggested above. Many epidemiologic studies have shown that smokers are at decreased risk for the development of neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease [reviewed in (31)]. In vitro model systems of Alzheimer’s disease have shown that nicotine-mediated protection of neuronal cells against β-amyloid-induced cytotoxicity is dependent upon Akt activation (32–34). Thus, would a therapeutic strategy against Akt tailored for lung cancer increase neuronal degeneration? Animal models may be useful to address this question.

Activation of Akt by nicotine or NNK clearly propagated the Akt pathway in NSCLC cells because increased phosphorylation of multiple identified and unidentified downstream substrates of Akt was observed in a time-dependent manner. Which substrate(s) might be most important for determining cellular responses to tobacco components? The patterns of phosphorylation of downstream substrates were mostly similar for nicotine or NNK, but two potentially important differences were that nicotine, but not NNK, increased phosphorylation of MDM2 and mTOR. Phosphorylation of MDM2 by Akt can promote nuclear translocation of MDM2 and degradation of p53, which can clearly affect apoptosis (35,36). mTOR can also promote cellular proliferation and cellular survival, especially in cells with high levels of Akt activity [reviewed in (37)]. Thus, propagation of the Akt signal to MDM2 or mTOR might be responsible for nicotine-induced survival. The availability of inhibitors of mTOR such as rapamycin will facilitate studies to determine the relative roles of these Akt substrates in nicotine-mediated survival.

The identification of nAchR subunits in NSCLC cell lines provides a mechanistic basis for Akt activation in response to tobacco components, and fills a gap in the analysis of nAchR subunit expression in cells derived from lung tissues. Once thought to be restricted to neuronal cells, nAchR subunit expression has now been demonstrated in human lung epithelial cells (12,16), SCLC cells (38), and NSCLC cells. In addition, nAchR have been described in keratinocytes (24) and endothelial cells. This wide distribution of nAchR may underlie the systemic physiological responses to smoking.

Although other studies have shown that nicotine or other agonists of nAchR such as acetylcholine can promote the growth of SCLC cells (25,39), our study is the first to demonstrate that nicotine or NNK stimulates NSCLC proliferation that is dependent upon Akt. The fact that expression of mutant Akt inhibited nicotine- or NNK-induced proliferation of H157 cells, suggests that other signaling pathways that might be increased by nicotine stimulation such as the MEK/ERK pathway (40) would not contribute greatly to proliferation in these cells unless they were directly upstream or downstream of Akt.

In addition to nAchR, NNK might also exert its biologic activity through activation of other cell surface receptors. NNK can indirectly stimulate DNA synthesis in NSCLC cells through stimulation of β-adrenergic receptors and the release of arachidonic acid (41), but the role of Akt was not assessed by these investigators. It may be noted that β-adrenergic antagonists did not block NNK-induced Akt activation in H157 or H1703 cells (data not shown). Thus, NNK-induced Akt activation and proliferation of H157 cells is probably due to stimulation of nAchR rather than β-adrenergic receptors. Regardless of the relative roles of different receptors at the cell surface, nicotine- or NNK-induced signaling converged on Akt, and proliferation was dependent upon Akt activation.

Although NNK served as a mitogen for H157 and H1703 cells, only nicotine served as a mitogen and an NFκB-dependent survival factor. In addition to the neuronal cell systems discussed above, nicotine can promote survival of many cell types including head and neck cancer cells (42,43). Nicotine can inhibit apoptosis induced by diverse stimuli such as administration of opioids, tumor necrosis factor, UV light or γ-radiation (42,44). In our studies, nicotine protected NSCLC cells against chemotherapy-induced apoptosis and serum deprivation-induced apoptosis. One mechanism by which nicotine protected NSCLC cells was through NFκB, because NSCLC cells expressing an inhibitor of NFκB, IκBα, were resistant to nicotine-mediated protection, and NFκB activity was directly stimulated by nicotine. These data are consistent with prior observations that cigarette smoke condensate (CSC) can increase NFκB activity (45). Given that cigarette smoke condensate is a complex mixture of ~4000 chemicals (46), we have identified a single component in this mixture, nicotine, as an NFκB-dependent survival factor. This may be relevant because increased expression of p50, a subunit of the NFκB complex, is observed in NSCLC tumors compared with normal lung tissues (47). Other mechanisms that could also contribute to nicotine-mediated survival include increased bcl-2 phosphorylation (48), but we were unable to detect phosphorylated bcl-2 in H157 cells (data not shown). The fact that NFκB mediates nicotine-induced NSCLC cellular survival suggests that new drugs that target the proteasome and NFκB such as bortezomib might mitigate possible detrimental effects of nicotine on chemotherapeutic responsiveness (49). Future studies will test this hypothesis.

Finally, these studies highlight the complex biology of nicotine and tobacco. Nicotine, originally thought only to be responsible for tobacco addiction, is now recognized for modulation of key cellular proteins, such as Akt, cyclin D1, NFκB and bcl-2, and key cellular processes, such as increased proliferation and survival, two components of the transformed phenotype (50). If the conditions used in these studies were to be replicated in vivo, it is hypothetically possible that increased growth of nascent, undiagnosed cancers and/or increased resistance to cytotoxic therapy could occur with exposure to nicotine. Because of the widespread availability and use of nicotine supplements in current or former smokers, further investigation on the sustained biological activities of nicotine is warranted.

Conflict of Interest Statement: None declared.
Supplementary material can be found at: http://www.carcin.oupjournals.org/.

References


Received July 9, 2004; revised March 10, 2005; accepted March 12, 2005