Cooperative Regulation of Non-Small Cell Lung Carcinoma by Nicotinic and Beta-Adrenergic Receptors: A Novel Target for Intervention

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Abstract

Lung cancer is the leading cause of cancer death; 80–85% of lung cancer cases are non-small cell lung cancer (NSCLC). Smoking is a documented risk factor for the development of this cancer. Although nicotine does not have the ability to initiate carcinogenic events, recent studies have implicated nicotine in growth stimulation of NSCLC. Using three NSCLC cell lines (NCI-H322, NCI-H441 and NCI-H1299), we identified the cooperation of nicotinic acetylcholine receptors (nAChRs) and β-adrenergic receptors (β-ARs) as principal regulators of these effects. Proliferation was measured by thymidine incorporation and MTT assays, and Western blots were used to monitor the upregulation of the nAChRs and activation of signaling molecules. Noradrenaline and GABA were measured by immunoassays. Nicotine-treated NSCLC cells showed significant induction of the α7nAChR and α4nAChR, along with significant inductions of p-CREB and p-ERK1/2 accompanied by increases in the stress neurotransmitter noradrenaline, which in turn led to the observed increase in DNA synthesis and cell proliferation. Effects on cell proliferation and signaling proteins were reversed by the α7nAChR antagonist α7-BTX or the β-blocker propranolol. Nicotine treatment also down-regulated expression of the GABA synthesizing enzyme GAD 65 and the level of endogenous GABA, while treatment of NSCLC cells with GABA inhibited cell proliferation. Interestingly, GABA acts by reducing β-adrenergic activated cAMP signaling. Our findings suggest that nicotine-induced activation of this autocrine noradrenaline-initiated signaling cascade and concomitant deficiency in inhibitory GABA, similar to modulation of these neurotransmitters in the nicotine-addicted brain, may contribute to the development of NSCLC in smokers. Our data suggest that exposure to nicotine either by tobacco smoke or nicotine supplements facilitates growth and progression of NSCLC and that pharmacological intervention by β blocker may lower the risk for NSCLC development among smokers and could be used to enhance the clinical outcome of standard cancer therapy.

Introduction

Lung cancer is the leading cause of cancer-related mortality in both men and women in the United States, with a similar trend documented globally. Industrialized regions such as North America and Europe have the highest rates [1]. The family of non-small cell lung cancers (NSCLC) consists of adenocarcinoma, squamous cell carcinoma and large cell carcinoma. NSCLC is highly resistant to existing cancer therapeutics, and the survival rate beyond 2 years is still discouraging despite recent advances in the development of novel agents that target epidermal growth factor or angiogenesis factors [2]. NSCLC accounts for about 80% of all lung cancer cases, with adenocarcinoma predominating. Smoking is a documented risk factor for NSCLC and is primarily responsible for the development of this cancer type in populations exposed to smoking [3,4]. The nitrosated nicotine-derived carcinogen 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butaneone (NNK) causes NSCLC in laboratory rodents [5,6] and has been identified as a high affinity agonist for nicotinic acetylcholine receptors (nAChRs) [7,8]. Cellular signaling in response to binding of NNK to nAChRs has been implicated in the growth regulation of NSCLC [9]. However, because the tobacco constituent nicotine is less carcinogenic compared to its potent carcinogenic derivative NNK, nicotine has attracted less interest for its role in smoking’s effects on cancer growth and stimulation. It has been earlier shown that nicotine stimulates angiogenesis via binding to α7 nAChR [10], a process that may stimulate tumor growth. However, these studies have addressed direct cellular responses to single doses of nicotine or NNK, which may be termed acute exposures. Such a protocol does not mimic real-life exposure where exposure to both agents in smokers is chronic, suggesting that similar to the effects of chronic nicotine in the brain, indirect mechanisms via the nAChR-mediated production of neurotransmitters may be involved. Moreover, nicotine replacement therapy may have adverse effects on the clinical outcome of NSCLC therapy via such mechanisms.

Nicotinic receptors are constituted of alpha subunits (homomorphic nAChRs) or a combination of alpha and beta subunits
physiological agonist for the Gα subunit upregulates the α7nAChR. It is thought that chronic exposure to nicotine increases the sensitivity of α7nAChR, from which adrenalin is formed enzymatically [13,14]. In the other hand, the heteromeric α7β2nAChR regulates the inhibitory neurotransmitter α-amino butyric acid (GABA) [15]. Chronic exposure to nicotine upregulates the expression of both receptors in the nervous system via posttranscriptional mechanisms without an increase in gene transcription [6,16,17]. The upregulation of α7β2nAChR protein is the response to nicotine-induced, long-term desensitization that reduces GABA production in the brain, an effect thought to cause nicotine addiction and craving [11]. By contrast, protein upregulation of the α7nAChR in the brain is not accompanied by long-term receptor desensitization, resulting in enhanced production of excitatory neurotransmitters [18].

It has been demonstrated that binding of nicotine to the α7nAChR stimulates colon cancer cells indirectly by increasing the production of noradrenaline, which in turn activated β-adrenergic receptor (β-AR) signaling [19]. Noradrenaline is the physiological agonist for the Gβγ-coupled β-ARs, and many of its biological effects are caused by the activation of adenyl cyclase downstream of Gβγ, which leads to the formation of intracellular cAMP [20,21]. Noradrenaline has strong stimulating effects, via beta-adrenergic receptor signaling, on a number of cancer types, including cancer of the colon [19,22,23], prostate [24], ovary [25], and pancreas [26]. It has also been shown that the selective β-adrenergic agonist isoproterenol stimulates DNA synthesis of human NSCLC cell lines [27]. A cooperative regulation seems to be at work, as NNK is not only an agonist for nAChRs [7,8] but also for β-ARs [28] and has been shown to stimulate proliferation and migration of human NSCLC cell lines in vitro via signaling effectors downstream of β-ARs. The beta-adrenergic signaling cascade activated by NNK in these cells included the adenyl cyclase/cAMP/CREB pathway as well as PKA-dependent transactivation of EGFR and its downstream effector, the ERK1/2 cascade [28-29]. Such activation of cancer stimulatory β-adrenergic signaling by NNK might also be the principal regulatory mechanism involved in the action of its parent compound, nicotine, because of its documented ability to cause the release of noradrenaline and adrenaline.

In the current study, three human NSCLC cell lines (NCI-H322, NCI-H441, NCI-H1299) were used to test the hypothesis that acute or chronic nicotine-induced modulation of α7nAChR and α7β2nAChR may contribute to the development and progression of AC in smokers in a manner similar to changes in these receptors in the nicotine-addicted brain, and that these effects can be neutralized by γ-amino butyric acid or a general beta-blocker such as propranolol.

**Materials and Methods**

**Cell lines and tissue culture**

The human NSCLC cell line NCI-H322 (histological subtype: adenocarcinoma with activating point mutation in K-ras) was purchased from the European Collection of Cell Cultures (Health Protection Agency, Porton Down, Salisbury, UK). The NSCLC cell lines NCI-H441 (histological subtype: adenocarcinoma without activating point mutation in K-ras) and NCI-H1299 cells (non-small cell lung carcinoma which lacks p53 protein expression, histological subtype not identified) were purchased from American Type Culture Collection (Manassas, VA, USA). Cell line NCI-H1299 was purchased by us 1 month earlier and therefore did not require authentication by us. Cell lines NCI-H322 and NCI-H441 were purchased by us several years earlier and were therefore authenticated by RADIL (Columbia, MO, USA) by species-specific PCR. Cells were maintained in RPMI-1640 culture medium (Gibco, Frederick, MD, USA) supplemented with fetal bovine serum (10% v/v), free of antibiotics at 37°C in an atmosphere of 5% CO₂ [29]. Similar to the majority of human NSCLCs [30], NCI-H322 carries k-ras point mutation while NCI-H441 does not have such a mutation.

**ELISA immunoassays for the detection of noradrenaline and GABA**

Neurotransmitter production mediated by nAChRs in response to single dose of nicotine was determined by acute exposure of cells for 30 minutes to 1 μM nicotine (nicotine [l]-tartrate, Sigma, St. Louis, MO, USA) in the presence or absence of the selective α7nAChR antagonist α-bungarotoxin (α-BTX, 200 nM, Calbiochem, Gibbstown, NJ, USA) or the selective α7β2nAChR antagonist N-n-decylnicotinium iodide (NDNI 200 nM, Sigma). Chronic exposure of cells with nicotine (1 μM) was conducted for 7 days in basal medium containing heat-inactivated horse serum (Sigma) as the only additive with replacement of nicotine every 24 hours. Control cells were maintained under identical conditions for 7 days without nicotine. In order to measure modulations in the sensitivity of α7nAChR and α7β2nAChR to chronic nicotine, control cells and nicotine pretreated cells were then washed with PBS and exposed for 30 minutes in basal medium to nicotine at concentrations from 10 nM through 10 μM, and noradrenaline and GABA were analyzed by immunoassays. For determination of EC₅₀ values, the data were analyzed by nonlinear regression and fitted to sigmoidal dose-response curves with variable slopes, using Prism GraphPad software (San Diego, CA, USA). For the nonlinear regression analysis of the ascending curves for noradrenaline, as a constraint for the calculation the EC₅₀ the values for the untreated control cells were entered as bottom of the curve (minimum). For the nonlinear regression analysis of the descending GABA curves, as a constraint for the calculation of the EC₅₀, the values for the untreated control cells were entered as top of the curve (maximum). The EC₅₀ values were tested for significant differences of control versus nicotine pretreated cells, using unpaired, two-tailed t tests.

**Assessment of cell proliferation by MTT assay**

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Sigma) was performed as previously described [31] to assess the concentration dependence of inhibitory effects of GABA on cell proliferation. NSCLC cells were entered as top of the curve (maximum). Absorbance was read with an ELISA reader at 450 nm. Quantification of the samples was achieved by comparing their absorbance with a reference curve prepared with known standard concentrations of noradrenaline and GABA. Data are expressed as mean values and standard errors from triplicate samples per treatment group. Statistical significance of data was assessed by one-way analysis of variance (ANOVA), the Tukey-Kramer multiple comparison test, and two-tailed, unpaired t test.
were treated with GABA (0.5 µM to 128 µM) for 72 hours. The data were then analyzed by nonlinear regression using GraphPad Prism software.

Also, an MTT assay was used to determine the effect of acute or chronic nicotine on NSCLC cells in the presence or absence of the general β-adrenergic antagonist (synonym: beta-blocker) propranolol (Sigma). The dose of nicotine used in this study was within the range of daily nicotine intake in heavy smokers [32].

**Determination of DNA synthesis by [3H]-thymidine incorporation assay**

Analysis of DNA synthesis by [3H]-thymidine incorporation assay was conducted as previously described [28]. Briefly, cells were seeded into 96-well plates (5 × 10^3 cells/well, triplicate wells/treatment group) in complete RPMI-1640 and allowed to settle; following 24-hour incubation in 37°C in an atmosphere of 5% CO₂ and 99% RH, complete medium was then replaced by fresh, low-serum medium (0.1% FBS) for 24-hour starvation, at the end of which medium was replaced by fresh, low-serum medium containing [3H]-thymidine (0.5 µCi/well) and treatment agents (inhibitor: 1 µM propranolol, or stimulator: 1 µM epinephrine, Sigma, or a combination of 1 µM propranolol for 10 minutes' preincubation followed by 1 µM epinephrine) or vehicle in a final volume of 200 µl/well. Following an additional incubation period of 24 hours in the presence of the treatment agents in 5% CO₂ and 99% RH at 37°C, cells were washed three times with PBS and lysed, adding 30 µl/well of 0.1 N NaOH. The plate was incubated at room temperature on a horizontal orbital microplate shaker. Free [3H]-thymidine was separated from incorporated [3H]-thymidine by vacuum filtration using a microplate harvester (Micromate 196, Packard, Meriden, CT, USA) onto backed glass fiber filters. The filters were flushed with isopropanol (200 µl/well) to fix adsorbed DNA and transferred to scintillation vials containing 3 ml counting cocktail (Bio-Safe-II, IL, USA). Radioactivity bound to the filters was measured by liquid scintillation spectrophotometry (Packard BioScience, Waltham, Massachusetts, USA). GraphPad software was used to analyze data. Means and standard errors were generated for count per minute. Significant differences between groups were assessed by ANOVA and unpaired Student t test.

**Protein expression analysis of nAChRs and their effectors by Western blotting**

To assess the effects of one-time nicotine exposure versus chronic exposure on the expression of nAChRs subunits (α7, α4), glutamate decarboxylase (GAD65), and the mitogen-activated protein kinases ERK1/2 or the cAMP response element binding protein CREB, NSCLC cells were seeded into tissue culture dishes (100 cm²) containing their growth media. When the cells had reached 60–65% confluence, they were rinsed once with 1 × PBS and starved of serum for 24 hours. Following removal of the media and replacement with fresh basal media, nicotine (1 µM) and cells were incubated for 30 minutes in serum-free media with heat-inactivated horse serum for 7 days. In another set of experiments, cells were seeded into 100 cm² culture dishes containing their growth media until they had reached 60–65% confluence, and then cells were switched into basal media for 24-hour starvation. Following starvation, fresh basal media was added containing the treatments (1 µM nicotine, 30 minutes; 1 µM propranolol, 40 minutes; or a combination of propranolol for 10 minutes followed by co-treatment with nicotine for 30 minutes). Following treatments, cells were then washed once with cold 1 × PBS followed by cell lystate collection in which the protein concentration was estimated by BCA Protein Assay (Pierce, Rockford, IL, USA). Western blotting procedure was conducted as previously described [26]. Following blocking, membranes were then incubated with primary antibodies overnight at 4°C. These antibodies included total CREB (Upstate Biotechnology, Lake Placid, NY, USA), p-CREB, p-ERK1/2 and ERK1/2 (Cell Signaling, Danvers, MA, USA), anti-α7nAChR, α7nAChR, GAD65 (Millipore, Billerica, MA, USA) and anti-beta-actin (Sigma). After incubation with horseradish peroxidase-labeled secondary antibody (anti-mouse or anti-rabbit, Cell Signaling) for 1 hour at room temperature, immunoreactive bands were detected by chemiluminescent reaction (ECL, Amersham Biosciences, Piscataway, NJ, USA) via autoradiography on Kodak BioMax XAR film. Relative densities of the bands were determined by image analysis using NIH SCION image analysis software. Mean values and standard errors from five densitometric readings per band were analyzed by one-way ANOVA and Tukey-Kramer multiple comparisons test or by non-parametric ANOVA and Mann-Whitney test as appropriate.

**Results**

NSCLC cells release the neurotransmitters noradrenaline and GABA in response to nicotine, and these effects are inhibited by α7 or α4 nAChR antagonists

Exposure of NCI-H322 cells to a single dose (1 µM) of nicotine for 30 minutes caused a significant (P<0.001) increase in noradrenaline production (Fig. 1). This response was significantly (P<0.001) inhibited by the selective α7nAChR antagonist α-BTX (200 nM; Fig. 1). At the same time, nicotine significantly (P<0.01) reduced GABA production, an effect reversed (P<0.01) by the selective α4β2nAChR antagonist NMDNI (Fig. 1).

**Figure 1. Effect of acute nicotine exposure (Nic, 1 µM for 30 minutes) on the production of noradrenaline and GABA in NCI-H322 cells.** Production of noradrenaline was significantly (P<0.001) increased (ELISA immunoassay), an effect inhibited by the α7nAChR antagonist α-BTX (200 nM for 30 minutes). Production of GABA was significantly (P<0.001) reduced by nicotine, a response blocked by the α4β2nAChR antagonist N-n-decylnicotinium iodide (NDNI, 200 nM). Columns are mean values and standard errors of triplicate samples per treatment group. doi:10.1371/journal.pone.0029915.g001
Exposures of NCI-H322 cells for 30 minutes to ascending concentrations of nicotine established clear dose-response curves (as indicated by goodness of fit R squared values between 0.9665 and 0.97320) for the stimulating effects of nicotine on noradrenaline production (Fig. 2A) and the suppressing effect on GABA release (Fig. 2B). The noradrenergic response was significantly increased in cells pretreated for 7 days with nicotine, as evidenced by a significantly lower EC$_{50}$ in the nicotine pretreated cells (EC$_{50}$ for unpretreated cells: 11.2±0.10 nM; EC$_{50}$ for nicotine pretreated cells: 1.2±0.11 nM). These findings indicate that the $\alpha_7$nAChR that regulates noradrenaline production was 9.2 times more sensitive to nicotine in cells pretreated for 7 days with nicotine than in unpretreated cells. In addition, the suppressing effects of nicotine on GABA production were significantly (P<0.0001) increased by chronic pre-exposure to nicotine (Fig. 2B), with EC$_{50}$ values of 11.2±0.83 nM and 1.4±0.81 nM, respectively. These findings indicate that the $\alpha_4\beta_2$nAChR was 7.9 times more desensitized by 7 days of nicotine exposure than by a single nicotine exposure for 30 minutes.

Acute or chronic exposure to nicotine increases, and GABA or propranolol inhibits cell proliferation

To assess the inhibitory effects of GABA on NCI-H322 cell proliferation, dose-response curves were established, using MTT assays. As exemplified in Fig. 3, the number of viable cells decreased with increasing concentrations of GABA, yielding an EC$_{50}$ of 2.3 μM (Fig. 3). By contrast, MTT assays in NCI-H441 and NCI-H1299 cells showed that acute (30 minutes) as well as chronic (7 days) exposure to nicotine (1 μM) significantly (P<0.0001) increased the number of viable cells with chronic exposure doubling this response (Fig. 4). These acute and chronic responses of both cell lines to nicotine were significantly (P<0.0001) inhibited by the broad-spectrum $\beta$-adrenergic antagonist propranolol (1 μM; Fig. 4). The difference between the group treated with propranolol alone versus the group treated with nicotine and propranolol in both cell lines were statistically significant (P<0.001; Fig. 4).

Exogenously-added epinephrine induces DNA synthesis in NSCLC cells

Determination of [$^3$H]-thymidine incorporation revealed a significant (P<0.001) increase in DNA synthesis in NCI-H441 cells (2.9-fold) when epinephrine (1 μM) was added to the culture medium and a 2-fold increase in NCI-H322 cells. This response was significantly (P<0.001) inhibited in both cell lines by the broad-spectrum $\beta$-adrenergic antagonist propranolol (1 μM; Fig. 5).

Nicotine enhances the expression of nAChR subunits, p-CREB and p-ERK while suppressing GAD65

Protein expression of $\alpha_7$nAChR in NCI-H322 cells increased 2.2 fold (P<0.001) after acute nicotine exposure and 2.7 fold after chronic exposure, while expression of $\alpha_4\beta_2$nAChR increased 1.8 (P<0.001) and 3.6 fold (P<0.001), respectively (Fig. 6A). Expression of the GABA-synthesizing enzyme GAD65 decreased to 0.7 and 0.3 fold, respectively, in cells treated with nicotine for 30 minutes or 7 days (Fig. 6A).

Protein analysis of the unphosphorylated and phosphorylated forms of CREB and ERK revealed significant (P<0.001) increases...
in the phosphorylation of both proteins in cells acutely exposed to nicotine, as opposed to chronically-exposed cells. The intensity of this response was particularly noteworthy for p-ERK, which showed a 6.6-fold increase in the nicotine pretreated cells compared with only a 2.2-fold increase in the unpretreated cells (Fig. 6B). On the other hand, NCI-H441 cells treated with 1 μM nicotine for 30 minutes showed a 2.75-fold increase in p-CREB (P<0.001) and a 2.9-fold increase in p-ERK1/2 (P<0.001) (Fig. 7A), while Western blot analysis of NCI-H1299 cells treated with 1 μM nicotine for 30 minutes revealed a 3.8-fold increase in p-CREB (P<0.001) and a 4.1-fold increase in p-ERK1/2 (P<0.001) (Fig. 7B). Both responses were significantly reduced by 1 μM propranolol (P<0.0001) in both cell lines, as illustrated in Fig. 7.

These findings are in accord with the observed nicotine-induced increase in the stimulatory noradrenergic response and decrease in inhibitory GABA response after chronic nicotine exposure, resulting in hyperactivity of CREB and ERK.

Discussion

Our data provide evidence, for the first time, that NSCLC cells produce their own stimulatory and inhibitory neurotransmitters and that these activities are regulated by nAChRs. The production of catecholamines by NSCLC cells observed in the current experiments is in accord with published observations that a significant number of NSCLC tumors and cell lines express the enzyme dopa-decarboxylase which mediates the conversion of L-Dopa to dopamine from which noradrenaline is formed [33]. The in vivo relevance of the current in vitro data is additionally supported by a recent report that nicotine-induced induction of NSCLC xenograft growth is reversed by the pharmacological...
The observed regulatory roles of the $\alpha_7nAChR$ for the production of noradrenaline and of the $\alpha_4\beta_2nAChR$ for the production of GABA by NSCLC cells are analogous to the function of these receptors in the brain where noradrenaline has excitatory effects, whereas GABA acts as the main inhibitory neurotransmitter [13,14]. Our data show that the $\alpha_7nAChR$-induced release of noradrenaline significantly stimulated NSCLC proliferation associated with the induction of p-ERK and p-CREB while the observed reversal of these effects by propranolol identify these and potentially other signaling proteins as the downstream effectors of $\beta$-ARs. The resulting cooperation of $\alpha_7nAChR$ and $\beta$-ARs represents an attractive novel target for the development of more effective intervention strategies for NSCLC.

Nicotine-induced stimulation of DNA synthesis and inhibition of apoptosis were first reported in neuroendocrine lung cancer cells [33,36]. Since then, numerous publications have described intracellular signaling in response to nAChR activation by nicotine or NNK and interpreted these effects as direct responses downstream of nAChRs [reviewed in 6]. However, a major function of nAChR-induced cation influx in the central nervous system is the regulation of excitatory and inhibitory neurotransmitters [6], suggesting that non-neuronal cells may respond in a similar fashion. This hypothesis is supported by our current data and is in accord with recent findings that hamsters with NNK-induced ACs have increased serum levels of noradrenaline and adrenaline accompanied by elevated cAMP in blood cells and tumor tissue [37]. Strong cancer-stimulating effects of noradrenaline and adrenaline, or stress that induces the release of these neurotransmitters from the adrenal medulla and sympathetic nerves, have also been demonstrated in cancer of the colon [22], prostate [24], mammary gland [38], and ovary [25,39].

Our data show that chronic exposure of NSCLC cells to nicotine modulated the expression and function of $\alpha_7nAChR$ and $\alpha_4\beta_2nAChR$ in a manner that enhanced the levels of cancer-stimulating noradrenaline while suppressing cancer-inhibiting GABA. These findings are in accord with the recently reported tumor suppressor function of GABA in NSCLC [40].

The observed upregulation of $\alpha_7nAChR$ protein in conjunction with increased stress neurotransmitters, cAMP, and induced p-
CREB and p-ERK1/2 in the current study indicate that chronic nicotine exposure rendered this receptor hyperactive. By contrast, upregulation in α4nAChR protein accompanied by suppression of GAD65 and GABA suggests that nicotine causes long-term desensitization of this receptor, resulting in a reactive upregulation of receptor protein. In support of this interpretation, our in vitro

Figure 7. Western immunoblot analyses in NCI-H441 (A) and NCI-H1299 cells (B). Nicotine (1 μM for 30 minutes) increased the expression of p-CREB (NCI-H441: 2.75 fold, P < 0.001; NCI-H1299: 3.8-fold, P < 0.001) and p-ERK1/2 (NCI-H441: 2.9 fold, P < 0.001; NCI-H1299: 4.1 fold, P < 0.001). Propranolol inhibited induction of these phosphorylated proteins (P < 0.001) in both cell lines. CREB and ERK1/2 were used as housekeeping proteins to ensure equal protein loading. Columns in the graph represent mean values and standard errors of five densitometric readings per band, expressed as a ratio of p-CREB over CREB or p-ERK over ERK1/2.

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dose-response curves with nicotine revealed an upregulated noradrenergic response and down-regulated GABA response in AC cells pre-exposed for 7 days to nicotine. The observed increase in noradrenergic response was significantly greater than the nicotine-induced increase in 7nAChR protein, indicating that 7nAChR was significantly sensitized to the agonist. Moreover, the significant decrease in GABA response observed in cells chronically exposed to nicotine supports the interpretation of a desensitized 4β2nAChR that regulates GABA production in these cells. Our findings are in accord with the “paradoxical” (without long-term desensitization) upregulation of 7nAChR as opposed to the desensitization-induced upregulation of 7β2nAChR in response to chronic nicotine reported in the brain [11,13] and demonstrate strong analogy to modulations of these receptors in the brain associated with nicotine addiction. In turn, the resulting hyperactivity of NSCLC-stimulating beta-adrenergic signaling suggests an important role of this cascade in the development and progression of this cancer in smokers. A recent electrophysiological study with immortalized human bronchial epithelial cells has shown that in vitro exposure to 1 μM nicotine for 48 hours significantly increased nicotine-induced currents [41], while investigations with immortalized human small airway epithelial cells have shown that exposure for 7 days to NNK caused a significant increase in the noradrenergic response of these cells to a nicotinic agonist [42]. Studies by PCR analyses have reported down-regulated gene expression of the 7nAChR subunit in the majority of investigated tissue samples from human ACs [43]. Although these studies were not accompanied by analyses of receptor protein expression that would detect posttranscriptional protein upregulation of the receptor in response to chronic nicotine [16,17], they provide additional support for a reduced function of 4β2nAChR in human AC.

NNK is an agonist for nAChRs [7] as well as β-ARs [28], and in vitro studies with NSCLC cells and small airway epithelial cells have shown that beta-adrenergic receptor activation by a synthetic agonist or NNK activated the adenylyl cyclase/cAMP/PKA/CREB cascade while at the same time trans-activating EGFR and its downstream effectors in a PKA-dependent manner [27,29,44]. Our current findings suggest that some of these signaling responses to NNK were triggered by the 7nAChR-activated release of noradrenaline. In addition, our findings suggest that nicotine-induced promotion of NSCLC growth reported in xenograft models [45] was at least in part caused by the nAChR-mediated release of noradrenaline and adrenaline into the systemic circulation. At the same time, production of endogenous GABA, which serves as the physiological inhibitor of this signaling cascade, was suppressed due to nicotine-induced, long-term desensitization of its regulatory 7β2nAChR. The simultaneous downregulation of GAD 65, as shown in our study, is in accord with observations that stress characterized by acetylcholine-induced activation of nAChR-mediated release of noradrenaline, adrenaline, and cortisol reduces GABA and GAB, resulting in an impaired GABA system [46]. While the mechanisms underlying these effects are poorly understood, the nicotine-induced increase in these stress neurotransmitters, accompanied by downregulation of GAD 65 and GABA levels in our study and their reversal by propranolol treatment is extremely encouraging, with potential for marker-guided prevention and adjuvant therapy of AC. This conclusion is supported by observations that propranolol prevented the development of NNK-induced NSCLC in hamsters that overexpressed the 7nAChR, P-CREB and P-ERK [47] while showing suppressed GAD expression [37], whereas epinephrine had strong tumor promoting effects [47]. The reversal of nicotine-induced effects by propranolol treatment in the current study is also in accord with reports that the posttranscriptional upregulation of nAChRs by nicotine is PKA dependent [48], as PKA is a substrate of cAMP formed downstream of β-ARs.

In summary, the cooperative regulation of NSCLC cells by nAChRs and β-ARs and the strong inhibiting effects of propranolol at several levels of these pathways identify agents that interfere with this regulatory cascade as promising new tools for marker-guided prevention and adjuvant therapy of a subset of NSCLCs.

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Author Contributions

Conceived and designed the experiments: HMS. Performed the experiments: HANA MHA. Analyzed the data: HMS HANA MHA. Wrote the paper: HANA HMS. Obtained funding: HMS.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, et al. (2007) Cancer statistics, 2007. CA Cancer J Clin 57: 43–66.
2. Bunn PA Jr, Thatcher N (2008) Systematic treatment for advanced (stage IIIB/IV) non-small cell lung cancer: more treatment options; more things to consider. Oncology 13 Suppl 1: 37–46.
3. Soh J, Toyooka S, Ichihara S, Asano H, Kobayashi N, et al. (2008) Sequential molecular changes during multistage pathogenesis of small peripheral adenocarcinomas of the lung J Thorac Oncol 3: 540–547.
4. Devesa SS, Bray F, Vrbová AP, Parkin DM (2005) International lung cancer trends by histologic type: male-female differences diminishing and adenocarcinoma rates rising. Int J Cancer 117: 294–299.
5. Schuller HM (2008) Neurotransmission and cancer: implications for prevention and therapy. Anticancer Drugs 19: 635–671.
6. Schuller HM (2009) Is cancer triggered by altered signalling of nicotinic acetylcholine receptors? Nat Rev Cancer 9: 195–205.
7. Schuller HM, Ofori M (1998) Tobacco-specific carcinogenic nitrosamines. Ligands for nicotinic acetylcholine receptors in human lung cancer cells. Biochem Pharmacol 55: 1377–1387.
8. Arredondo J, Chernyavsky AI, Grande SA (2006) Nicotinic receptors mediate tumorigenic action of tobacco-derived nitrosamines on immortalized oral epithelial cells. Cancer Biol Ther 5: 511–517.
9. West KA, Linnoila IR, Belinsky SA, Harris CC, Dennis PA (2004) Tobacco carcinogen-induced cellular transformation increases activation of the phosphotidlyinositol 3'-kinase/Akt pathway in vitro and in vivo. Cancer Res 64: 446–451.
10. Wu JC, Chrusciak A, De Jesus Perez VA, Singh H, Pintinou M, et al. (2009) Cholinergic modulation of angiogenesis: role of the 7 nicotinic acetylcholine receptor. J Cell Biochem 108: 433–446.
11. Lindstrom J, Anand R, Gerzanich V, Peng X, Wang F, et al. (1996) Structure and function of neuronal nicotinic acetylcholine receptors. Prog Brain Res 109: 125–137.
12. Wessler I, Kirkpatrick CJ (2008) Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. Br J Pharmacol 154: 1538–1571.
13. Barik J, Wonnacott S (2006) Indirect modulation by alpha7 nicotinic acetylcholine receptors of noradrenaline release in rat hippocampal slices: interaction with glutamate and GABA systems and effect of nicotine withdrawal. Mol Pharmacol 69: 618–628.
14. Mozayan M, Lee TJ (2007) Statins prevent cholinesterase inhibitor blockade of sympathetic alpha7-nAChR-mediated currents in rat superior cervical ganglion neurons. Am J Physiol Heart Circ Physiol 295: H1737–1744.
15. Markou A (2008) Review. Neurobiology of nicotine dependence. Philos Trans R Soc Lond B Biol Sci 363: 3139–3168.
16. Harkness PC, Milne NS (2002) Changes in configuration and subcellular distribution of alpha4beta2 nicotinic acetylcholine receptors revealed by chronic nicotine treatment and expression of subunit chimeras. J Neurosci 22: 10172–10181.
17. Marks MJ, Pauly JR, Gross SD, Deenihan ES, Hermans-Borgmeyer I, et al. (1992) Nicotine binding and nicotine receptor subunit RNA after chronic nicotine treatment. J Neurosci 12: 2763–2774.
18. Kawai H, Berg DR (2001) Nicotinic acetylcholine receptors containing alpha 7 subunits on rat cortical neurons do not undergo long-lasting inactivation even when up-regulated by chronic nicotine exposure. J Neurochem 70: 1367–1378.
19. Wong HP, Yu L, Lam EK, Tai EK, Wu WK, et al. (2007) Nicotine promotes cell proliferation via alpha7-nicotinic acetylcholine receptor and catecholamine-synthesizing enzymes-mediated pathway in human colon adenocarcinoma HT-29 cells. Toxicol Appl Pharmacol 221: 261–267.
20. Ruffolo RR Jr., Boudinell W, Hieble JP (1990) Alpha- and beta-adrenoceptors: from the gene to the clinic. 2. Structure-activity relationships and therapeutic applications. J Med Chem 33: 3681–3716.
21. Hoffman BB, Leffowitz RJ (1996) Catecholamines and sympathomimetic drugs. In: Goodman Gilman A, Rall TW, Nies AS, Taylor P, eds. The pharmacological basis of therapeutics. New York: Pergamon Press. pp 187–220.
22. Masur K, Niggemann B, Zanker KS, Entscheid F (2001) Norpapinephrine-induced migration of SW 480 colon carcinoma cells is inhibited by beta- blockers. Cancer Res 61: 2866–2869.
23. Wong HP, Yu L, Lam EK, Tai EK, Wu WK, et al. (2007) Nicotine promotes colon tumor growth and angiogenesis through beta-adrenergic activation. Toxicol Sci 97: 279–297.
24. Palm D, Lang K, Niggemann B, Drell TL, 4th, Masur K, et al. (2006) The norpapinephrine-driven metastasis development of PC-3 human prostate cancer cells in BALB/c nude mice is inhibited by beta-blockers. Int J Cancer 118: 2744–2749.
25. Sood AK, Bhatty R, Kamat AA, Landen CN, Han L, et al. (2006) Stress hormone-mediated invasion of ovarian cancer cells. Clin Cancer Res 12: 369–375.
26. Al-Wadei HA, Plummer HK, 3rd, Schuller HM (2009) Nicotine stimulates pancreatic cancer xenografts by systemic increase in stress neurotransmitters and suppression of the inhibitory neurotransmitter gamma-aminobutyric acid. Carcinogenesis 30: 506–511.
27. Park PG, Merryman J, Orloff M, Schuller HM (1995) Beta-adrenergic mitogenic signal transduction in peripheral lung adenocarcinoma: implications for individuals with preexisting chronic lung disease. Cancer Res 55: 3504–3508.
28. Schuller HM, Tithof PK, Williams M, Plummer H, 3rd (1999) The tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone is a beta-adrenergic agonist and stimulates DNA synthesis in lung adenocarcinoma via beta-adrenergic receptor-mediated release of arachidonic acid. Cancer Res 59: 4510–4515.
29. Laag E, Majidi M, Cokanova M, Masl T, Takahashi T, et al. (2006) NNK activates ERK1/2 and CREB/ATF-1 via beta-1-AR and EGFR signaling in human lung adenocarcinoma and small airway epithelial cells. Int J Cancer 119: 1547–1552.
30. Mitoudomi T, Viallet J, Mulshine JL, Linnolla RI, Minna JD, et al. (1991) Mutations of ras genes distinguish a subset of non-small-cell lung cancer cell lines from small-cell lung cancer cell lines. Oncogene 6: 1333–1362.
31. Al-Wadei HA, Takahashi T, Schuller HM (2006) Growth stimulation of human pulmonary adenocarcinoma cells and small airway epithelial cells by betacarotene via activation of cAMP, PKA, CREB and ERK1/2. Int J Cancer 118: 1370–1380.
32. Lawson GM, Hurt RD, Dale LC, Olford KP, Crogan IT, et al. (1998) Application of serum nicotine and plasma cotinine concentrations to assessment of nicotine replacement in light, moderate, and heavy smokers undergoing transdermal therapy. J Clin Pharmacol 38: 502–509.
33. Gazdar AF, Helma Lj, Israel MA, Russell EK, Linnolla RI, et al. (1980) Expression of neuroendocrine cell markers L-Dopa Decarboxylase, chromogranin A, and dense core granules in human tumors of endocrine and nonendocrine origin. Cancer Res 40: 4078–4082.
34. Al-Wadei HA, Al-Wadei MH, Ullah MF, Schuller HM (2011) Gamma-amino butyric Acid inhibits the nicotine-induced stimulatory challenge in xenograft models of non-small cell lung carcinoma. Cancer Drug Target:In press.
35. Schuller HM (1989) Cell type specific, receptor-mediated modulation of growth kinetics in human lung cancer cell lines by nicotine and tobacco-related nitrosamines. Biochem Pharmacol 38: 3439–3442.
36. Maneckjee R, Minna JD (1990) Opioid and nicotine receptors affect growth regulation of human lung cancer cell lines. Proc Natl Acad Sci U S A 87: 3294–3298.
37. Al-Wadei HA, Schuller HM (2009) Nicotinic receptor-associated modulation of stimulatory and inhibitory neurotransmitters in NNK-induced adenocarcinoma of the lungs and pancreas. J Pathol 218: 437–445.
38. Drell TL, 4th, Joseph J, Lang K, Niggemann B, Zanker KS, et al. (2003) Effects of neurotransmitters on the chemokinesis and chemotaxis of MDA-MB-468 human breast carcinoma cells. Breast Cancer Res Treat 80: 63–70.
39. Thaker PH, Han LY, Kumat AA, Arevalo JM, Takahashi R, et al. (2006) Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. Nat Med 12: 939–944.
40. Schuller HM, Al-Wadei HA, Majidi M (2008) Gamma-aminobutyric acid, a potential tumor suppressor for small airway-derived lung adenocarcinoma. Carcinogenesis 29: 1979–1985.
41. Fu XY, Landstrom J, Spindel ER (2009) Nicotine activates and up-regulates nicotinic acetylcholine receptors in bronchial epithelial cells. Am J Respir Cell Mol Biol 41: 93–99.
42. Al-Wadei HA, Al-Wadei MH, Masl T, Schuller HM (2010) Chronic exposure to estrogen and the tobacco carcinogen NNK cooperatively modulates nicotinic receptors in small airway epithelial cells. Lung Cancer 69: 33–39.
43. Lam DC, Girard L, Ramirez R, Chau WS, Suen WS, et al. (2007) Expression of nicotinic acetylcholine receptor subunit genes in non-small-cell lung cancer reveals differences between smokers and nonsmokers. Cancer Res 67: 4638–4647.
44. Majidi M, Al-Wadei HA, Takahashi T, Schuller HM (2007) Nongenomic beta-2 estrogen receptors enhance beta 1 adrenergic signaling induced by the nicotine-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in human small airway epithelial cells. Cancer Res 67: 6863–6871.
45. Davis R, Rizwani W, Banerjee S, Kowacs M, Haura E, et al. (2009) Nicotine promotes tumor growth and metastasis in mouse models of lung cancer. PLoS One 4: e7524.
46. Hu W, Zhang M, Czeb H, Flug G, Zhang W (2010) Stress impairs GABAergic network function in the hippocampus by activating nongenomic glucocorticoid receptors and affecting the integrity of the parvalbumin-expressing neuronal network. Neuropsychopharmacology 35: 1695–1707.
47. Schuller HM, Porter B, Riechert A (2000) Beta-adrenergic modulation of NNK-induced lung carcinogenesis in hamsters. J Cancer Res Clin Oncol 126: 624–630.
48. Govind AP, Vezina P, Green WN (2009) Nicotine-induced upregulation of nicotinic receptors: underlying mechanisms and relevance to nicotine addiction. Biochem Pharmacol 78: 736–765.