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Nitrosamines as nicotinic receptor ligands

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Abstract

Nitrosamines are carcinogens formed in the mammalian organism from amine precursors contained in food, beverages, cosmetics and drugs. The potent carcinogen, NNK, and the weaker carcinogen, NNN, are nitrosamines formed from nicotine. Metabolites of the nitrosamines react with DNA to form adducts responsible for genotoxic effects. We have identified NNK as a high affinity agonist for the $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) whereas NNN bound with high affinity to epibatidine-sensitive nAChRs. Diethylnitrosamine (DEN) bound to both receptors but with lower affinity. High levels of the $\alpha 7$ nAChR were expressed in human small cell lung cancer (SCLC) cell lines and in hamster pulmonary neuroendocrine cells (PNECs), which serve as a model for the cell of origin of human SCLC. Exposure of SCLC or PNECs to NNK or nicotine increased expression of the $\alpha 7$ nAChR and caused influx of Ca^{2+} , activation of PKC, Raf-1, ERK1/2, and c-myc, resulting in the stimulation of cell proliferation. Signaling via the $\alpha 7$ nAChR was enhanced when cells were maintained in an environment of 10–15% CO_2 similar to that in the diseased lung. Hamsters with hyperoxia-induced pulmonary fibrosis developed neuroendocrine lung carcinomas similar to human SCLC when treated with NNK, DEN, or nicotine. The development of the NNK-induced tumors was prevented by green tea or theophylline. The beta-adrenergic receptor agonist, isoproterenol or theophylline blocked NNK-induced cell proliferation in vitro. NNK and nicotine-induced hyperactivity of the $\alpha 7$ nAChR/RAF/ERK1/2 pathway thus appears to play a crucial role in the development of SCLC in smokers and could be targeted for cancer prevention.

Keywords

Tobacco nitrosamines; nicotinic acetylcholine receptor; small cell lung cancer; pulmonary neuroendocrine cell

Introduction

Nitrosamines are formed by the nitrosation of simple amine precursors in the mammalian organism. They are characterized by the presence of the N-nitroso group and may be aliphatic or ring structures (Figure 1). The precursors of nitrosamines are almost ubiquitous in the environment, as they are contained in numerous foods, beverages, cosmetics, and medicines. Among the best-studied members of this family of agents are the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosornicotine (NNN) and N-nitrosodiethylamine (DEN), which is found in tobacco products as well as other

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environmental sources (Figure 2). Nitrosamines are metabolically converted via oxidative enzymes into a number of metabolites, some of which bind to the DNA molecule to form adducts associated with activating point mutations in K-ras and inactivating mutations in p53, both of which are found in numerous human cancers (Hecht, 1996; Hoffmann, Rivenson and Hecht, 1996; Wogan, Hecht, Felton, Conney and Loeb, 2004). Regardless of their route of administration, NNK, NNN, and DEN cause lung cancer in all laboratory rodents tested, with NNK being the most potent and NNN the weakest of these three carcinogens. In light of the strong etiological association of smoking with lung cancer, the tobacco nitrosamines are thought to be primarily responsible for the high lung cancer burden in individuals who smoke.

Nicotine, from which NNK and NNN are formed by nitrosation in the mammalian organism and during the processing of tobacco, is an agonist for the family of nicotinic acetylcholine receptors, which are expressed in the majority of mammalian cells. The affinity of nicotine to these receptors is greater than that of their physiological agonist, the neurotransmitter acetylcholine.

Accordingly, nicotine displaces acetylcholine from these receptors. The resulting hyper stimulation of nicotinic receptor pathways is responsible for the diverse biological effects of nicotine. The biology of nicotinic acetylcholine receptors (nAChRs) and the interaction of nicotine with these receptors have been extensively studied in the central nervous system as well as muscle tissues. It has thus been shown that nAChRs are comprised of a central ion channel with high permeability for Ca^{2+} enclosed by subunits termed alpha through delta. Neuronal nAChRs may contain α_2 through α_9 and β_2 through β_4 subunits whereas the muscle nAChRs contain additionally gamma and delta subunits (Gotti, Fornasari and Clementi, 1997; Wessler, Kirkpatrick and Racke, 1998). In addition to the central nervous system and peripheral nerves of the vagus, nAChRs with subunit compositions of neuronal nAChRs have also been identified in a large number of non-neuronal cells in peripheral organs (Grando, Kawashima and Wessler, 2003).

A potential role of nAChRs in smoking-associated lung carcinogenesis was first suggested by a publication from our laboratory in 1989, which showed that nicotine and NNK stimulated the proliferation of cell lines derived from human small cell lung cancer (SCLC) when the cells were maintained in an environment of 10% CO_2 and that this response was blocked by the neuronal nAChR antagonist hexamethonium (Schuller, 1989). Two other laboratories subsequently reported nicotine-evoked stimulation of cell proliferation (Cattaneo, Codignola, Vicentini, Clementi and Sher, 1993) or inhibition of apoptosis (Maneckjee and Minna, 1990; Maneckjee and Minna, 1994) in human SCLC cell lines. The initial findings from these three laboratories suggested that nicotine itself may contribute to the development of smoking-associated SCLC and that the nicotine-derived nitrosamine NNK may interact with nicotinic receptor signaling. This review summarizes our current knowledge on the interaction of carcinogenic nitrosamines with nicotinic receptors and their implications for lung cancer as reported in the Second International Symposium on Non-Neuronal Acetylcholine (Mainz, Germany, 2006).

Ligand Binding of nitrosamines to nicotinic acetylcholine receptors

The tobacco-specific nitrosamines NNN and NNK are nitrosated nicotine derivatives. NNN closely resembles nicotine in structure whereas the formation of NNK from nicotine involves opening of the pyrrolidine ring (Figure 2). DEN, which is found in tobacco as well as numerous foods, beverages, and cosmetics, has some structural similarity with the physiological agonist for nAChRs, acetylcholine (Figure 2). To test the hypothesis that these three nitrosamines bind to members of the nAChR family, we used the classic pharmacological tool for the identification of ligand binding to a receptor, radio-ligand binding assays. As the biological

test systems for these assays, we selected cell lines derived from the two leading types of smoking-associated lung cancer, SCLC and pulmonary adenocarcinoma (PAC). Some of these assays were additionally performed in cultured hamster pulmonary neuroendocrine cells, which serve as a model for the cell of origin of human SCLC. Taking advantage of a report that had identified the alpha bungarotoxin (α BTX)-sensitive nAChR comprised of homomeric $\alpha 7$ subunits ($\alpha 7$ nAChR) as the mediator of nicotine-induced cell proliferation in human SCLC cells (Cattaneo, Codignola, Vicentini, Clementi and Sher, 1993), we assessed binding of NNK, NNN, and DEN to this receptor in the human SCLC cell lines NCI-H69 and NCI-H82. Saturation binding assays with ascending concentrations of [125 I] α -BTX and analysis of the binding data by nonlinear regression for single-site isotherms identified a $B_{\max}$ of 17.69 fmol/mg protein, suggestive of a relatively high level of expression of $\alpha 7$ nAChRs in these cells (Schuller and Orloff, 1998). Saturation binding assays with [125 I] α BTX in fetal hamster pulmonary neuroendocrine cells revealed a similar $B_{\max}$ (15.5 fmole/mg protein) in these cells (Plummer, Sheppard and Schuller, 2000). By contrast, no binding of this radio-ligand was detected at the concentrations of [125 I]BTX used (1 pM-100mM) in the two human lung adenocarcinoma cell lines, NCI-H322 and NCI-H441 (Schuller and Orloff, 1998), suggesting the absence of significant numbers of functional $\alpha 7$ nAChRs in these cells. When ascending concentrations of nicotine, NNK, NNN (Schuller and Orloff, 1998), or DEN competed with [125 I] α BTX (20 nM) for the $\alpha 7$ nAChR binding sites in the SCLC cell lines, all four agents bound to the receptor, with NNK and DEN showing the highest affinities (Figure 3). Analysis of the binding data by nonlinear regression for a single class of receptors identified EC_{50} values of 0.03 and 0.3 μ M, respectively, for NNK and DEN as opposed to 40.4 μ M for nicotine and 93 μ M for NNN. The affinity to the $\alpha 7$ nAChR of NNK was thus 1,347 times and that of DEN 134.7 times higher than that of nicotine whereas the affinity of nicotine to this receptor was about 2,3 times greater than that of NNN. Similar EC_{50} values for nicotine and NNK in competition for $\alpha 7$ nAChR sites with [125 I] α BTX were calculated by linear regression analysis from binding data of assays generated in fetal hamster pulmonary neuroendocrine cells (Plummer, Sheppard and Schuller, 2000). The simultaneous exposure to all four agents during smoking will thus likely result in ligand binding of NNK and DEN to the $\alpha 7$ nAChR while binding of nicotine or NNN would only be expected with very high concentrations of these agents. Saturation binding assays with ascending concentrations of [3 H]epibatidine, which binds preferentially to the $\alpha 2$ - $\alpha 6$ subunits of nAChRs, revealed significant binding of this ligand in the PAC cell lines NCI-H322 and NCI-H441 (Schuller and Orloff, 1998). Analysis of the binding data by nonlinear regression identified a high $B_{\max}$ value of 158.4 fmole/mg protein, suggestive of high levels of epibatidine-sensitive nAChRs in these cells. By contrast, no binding of [3 H]epibatidine at the concentrations used (1 pM-100 mM) was detected in the two SCLC cell lines (Schuller and Orloff, 1998), indicating the absence of significant numbers of functional epibatidine-sensitive nAChRs in these cells. Competition binding assays in which nicotine, NNK, NNN (Schuller and Orloff, 1998), or DEN competed with [3 H]epibatidine for nicotinic receptor binding sites in the PAC cell lines revealed binding of all four agents to epibatidine-sensitive nAChRs (Figure 3). Analysis of the binding data by nonlinear regression for single-site isotherms revealed an exceptionally high affinity of NNN to this receptor (EC_{50} : 0.46 nM). The EC_{50} values for nicotine (1.7 μ M), DEN (6.6 μ M) and NNK (17.3 μ M) were all significantly greater, indicating lower affinity to these receptors. The affinity of NNN to epibatidine-sensitive nAChRs was thus about 3,700 times higher than that of nicotine (Schuller and Orloff, 1998). Upon simultaneous exposure to these four agents in a smoker, NNN is therefore highly likely to bind to epibatidine-sensitive nAChRs while the other three agents would only be expected to bind to these receptors when their concentrations are very high. In accord with the fact that the $\alpha 7$ nAChR is an ion channel with high permeability for Ca^{2+} , flow cytometric studies with human SCLC cell lines NCI-H69 and NCI-H82 demonstrated influx of Ca^{2+} associated with opening of voltage-gated Ca^{2+} -channels of the L, N, and P types in response to NNK, an effect blocked by α BTX (Sheppard, Williams, Plummer and Schuller, 2000). These findings identified NNK as an agonist for the $\alpha 7$ nAChR.

The high affinity of NNK to the $\alpha 7$ nAChR and of NNN to epibatidine-sensitive nAChRs observed by us in human SCLC and PAC cell lines, respectively, was also reported in human oral keratinocytes and bronchial epithelial cells during this symposium by Dr. Grando (Arredondo, Chernyavsky, Jolkovsky, Pinkerton and Grando, 2006a). The concentration of nicotine in the mainstream smoke of cigarettes is on average 5,000–10,000 times higher than that of NNK and about 2,000 to 3,000 times higher than that of NNN (Hecht and Hoffmann, 1988). The prevalence of nicotine over NNK in tobacco products is counteracted by the significantly higher affinity of NNK to the $\alpha 7$ nAChR and the higher affinity of nicotine to epibatidine-sensitive receptors, which are considerably more numerous in mammalian organs, including the lungs. Upon simultaneous exposure to nicotine and NNK, the nitrosamine will therefore likely bind to $\alpha 7$ nAChRs while nicotine binds to epibatidine-sensitive nAChRs. In addition, the extremely high affinity of NNN to epibatidine-sensitive nAChRs will lead to binding of this nitrosamine to this receptor family in smokers while nicotine will only bind to these receptors if binding sites are still available after NNN binding has saturated. In addition, available figures on relative exposure levels of NNN, NNK, and nicotine in smokers are based on the concentration of these agents in tobacco products and tobacco smoke while not including the amounts of nitrosamines formed endogenously from nicotine in the mammalian organism. These amounts can be substantial and can also show considerable inter-individual variations, pending on the presence of other factors that facilitate the endogenous nitrosation of nicotine, such as non-tobacco nitrosating agents. Collectively, these findings suggest that many of the biological effects that have been reported as the result of exposure to nicotine in smokers are in reality caused by interaction of nitrosamines with nAChRs.

Nitrosamine-induced signaling via nAChRs and lung cancer

Lung cancer is commonly classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC is comprised of neuroendocrine cells that express neuroendocrine markers such as L-dopa decarboxylase, serotonin, mammalian bombesin, calcitonin, neuron-specific enolase, and others (Gazdar, Helman, Israel, Russell, Linnoila, Mulshine, Schuller and Park, 1988). SCLC frequently demonstrates amplification of c-myc and harbors mutations in the retinoblastoma and p53 genes, while not expressing point mutations in k-ras (Salgia and Skarin, 1998). Pulmonary neuroendocrine cells (PNECs), which function as hypoxia-sensitive chemoreceptors in the lungs (Cutz and Jackson, 1999), share numerous phenotypic and functional features with SCLC cells and are the putative cell type of origin of this lung cancer. Any lung cancer that cannot be classified as SCLC is diagnosed as NSCLC, a heterogeneous family of cancers. The three major histological NSCLC types are adenocarcinoma, squamous cell carcinoma and large cell carcinoma, with adenocarcinoma predominating. Contrary to SCLC, these cancers do not express amplification in c-myc. By contrast, activating point mutations in k-ras are common as are inactivating mutations in p53 (Mitsudomi, Viallet, Mulshine, Linnoila, Minna and Gazdar, 1991; Salgia and Skarin, 1998). Squamous cell carcinomas are thought to arise from the pseudostratified epithelial lining comprised of basal cells, ciliated cells and mucous cells of large airways. By contrast, most adenocarcinomas are believed to originate from the simple epithelial lining comprised of Clara cells and ciliated cells of small airways while a small subset of these cancers may also arise from alveolar epithelial type II cells. Smoking is the single best-identified risk factor for all lung cancers. In addition, exposure to ionizing radiation and chloromethyl ethers, both of which cause pulmonary fibrosis, further enhance the risk of smokers to develop SCLC (Cook, Miller and Bunn, 1993; Devesa, Bray, Vizcaino and Parkin, 2005).

Some of our early studies have shown that NNK, DEN and nicotine stimulate the proliferation of human lung cancer cells with neuroendocrine features, an effect blocked by the broad-spectrum antagonist for neuronal nAChRs, hexamethonium (Schuller, 1989). To further elucidate the cellular signaling involved, we used NNK-exposed SCLC cells and fetal hamster

PNECs, with nicotine-exposed cells (1 μ M) serving as positive controls. Using in vitro kinase Raf-1 activation assays and Western blotting, we found that a very low concentration of only 100 pM NNK caused significant activation of Raf-1 and phosphorylated the extracellular signal regulated kinases ERK1/2 in both cell systems (Jull, Plummer and Schuller, 2001). These effects were completely blocked by α BTX (20 nM), by the PKC inhibitor sphingosine (10 μ M), or by the serotonin uptake inhibitor, imipramine (1 μ M). The addition of serotonin (100 pM) to the cultured cells mimicked the effects of nicotine or NNK on Raf-1/ERK1/2 activation and cell proliferation (Schuller, Jull, Sheppard and Plummer, 2000), suggesting that the autocrine growth factor serotonin mediated some of these responses. In support of this interpretation, analysis by enzyme immunoassays of serotonin released into the culture medium detected significant release of this biogenic amine in cells treated with nicotine or NNK. Exposure of the cells to 10% CO₂, as opposed to the standard environment of 5% CO₂, used to culture these cells also caused serotonin release and enhanced their responsiveness to NNK or nicotine (Plummer, Sheppard and Schuller, 2000; Schuller, 1998). Cell proliferation analyzed by [³H]thymidine incorporation assays correlated with the observed signaling responses, with significant stimulation of DNA synthesis in SCLC cells and PNECs exposed to 100 pM NNK or 1 μ M nicotine that was enhanced by 10% CO₂ and completely inhibited by α BTX, imipramine, sphingosine or the MEK inhibitor PD98059 (Jull, Plummer and Schuller, 2001; Plummer, Sheppard and Schuller, 2000; Schuller, 1994; Schuller, 1998). In addition, NNK-induced proliferation of SCLC cells was significantly reduced by the beta-adrenergic receptor agonist, isoproterenol, presumably via cAMP-induced inhibition of Raf (Plummer, Dhar, Cekanova and Schuller, 2005). NNK also phosphorylated the transcription factor c-myc in a time-dependent manner (Jull, Plummer and Schuller, 2001). This finding has recently been extended by another laboratory that reported growth-promoting cooperation of Bcl2 and c-myc via phosphorylation at specific sites by NNK in SCLC cells (Jin, Gao, Flag and Deng, 2004). In addition, these investigators showed that NNK induced ERK1/2-dependent phosphorylation of μ - and m -calpains as well as the secretion of these calpains, and that these effects stimulated cell migration and invasion (Xu and Deng, 2004). However, in the absence of control experiments with serotonin uptake inhibitors, it is not clear if the observed phosphorylations of Bcl2, c-myc, or calpains were mediated via signaling of the α 7nAChR or indirectly in response to nAChR-mediated serotonin release and re-uptake.

Contrary to many other cell surface receptors, which are downregulated by chronic exposure to agonists, the α 7nAChR is upregulated in smokers and after experimental exposure to nicotine (Peng, Gerzanich, Anand, Wang and Lindstrom, 1997). In analogy to these reports, NNK upregulated the expression of α 7nAChR mRNA in hamster PNECs (Plummer, Sheppard and Schuller, 2000). These findings suggest that NNK significantly contributes to the “paradoxical upregulation” of the α 7nAChR observed in smokers.

Collectively, these findings suggest that NNK and nicotine induce mitogenic signaling in SCLC and PNECs via PKC, Raf-1, and c-myc (Figure 4), and is consistent with the frequent amplification of c-myc in SCLC. The strong inhibitory effects of the serotonin uptake inhibitor imipramine on NNK and nicotine-induced signaling and cell proliferation and the mitogenic response to exogenously added serotonin suggest that a significant proportion of the observed NNK or nicotine-induced cellular signaling responses were not directly mediated by nAChR signaling but were rather caused by the α 7nAChR-stimulated release and re-uptake of serotonin, which is an autocrine growth factor for SCLC and PNECs. This interpretation is in accord with findings by another laboratory that reported the stimulation of an autocrine serotonergic loop in SCLC cells by nicotine (Cattaneo, Codignola, Vicentini, Clementi and Sher, 1993). The observed enhancing effects of an environment of 10% CO₂ in the incubator on responses to nicotine and NNK was a particularly interesting finding and indicates that SCLC cells have retained their ability to act as chemoreceptors for hypoxia, a function originally described for their putative cells of origin, PNECs (Cutz and Jackson,

1999;Youngson, Nurse, Yeger and Cutz, 1993). Hyperplasia of PNECs is a characteristic response to chronic non-neoplastic pulmonary diseases, such as bronchitis/bronchiolitis, emphysema, chronic obstructive pulmonary disease (COPD), or fibrosis associated with impaired pulmonary oxygenation and increased CO₂ concentration (Gosney, 1992). Dr. Cutz's laboratory has shown that the ability of PNECs to sense decreases in O₂ concentration is mediated by a receptor that operates as an ion channel and stimulates the release of serotonin to modulate airway tonus (Cutz and Jackson, 1999;Youngson, Nurse, Yeger and Cutz, 1993). Our data suggest that the hypoxia receptor cooperates with the $\alpha 7$ nAChR in the regulation of serotonin release and cell proliferation (Figure 4). In fact, we have shown that introduction of the SCLC cell lines NCI-H69 and NCI-H82 into an atmosphere of 10% CO₂ rapidly activated ERK1/2 and p70 ribosomal S6 kinase, leading to a significant stimulation of DNA synthesis (Merryman, Park and Schuller, 1997). Interestingly, the observed responses to elevated CO₂ were selective for the tested SCLC cells whereas two lung adenocarcinoma cell lines and a squamous cell carcinoma cell line responded with a decrease in cell proliferation and a large cell carcinoma cell line showed no response (Merryman, Park and Schuller, 1997). These findings imply that preexisting pulmonary diseases that impair the oxygenation of the lungs may selectively promote the development of SCLC. In support of this interpretation, NNK (Schuller, Witschi, Nylen, Joshi, Correa and Becker, 1990) or DEN (Reznik, Reznik-Schuller and Mohr, 1977;Reznik-Schuller, 1976) caused the development of lung adenocarcinomas when administered to healthy animals. By contrast, we found that hamsters with hyperoxia-induced pulmonary fibrosis developed neuroendocrine carcinomas when treated with NNK (Schuller, Witschi, Nylen, Joshi, Correa and Becker, 1990) or DEN (Schuller, Becker and Witschi, 1988). Even nicotine, which has been extensively tested in laboratory animals and was found to be non-carcinogenic, induced neuroendocrine lung carcinomas in a small but significant number of hamsters with hyperoxia-induced pulmonary fibrosis (Schuller, McGavin, Orloff, Riechert and Porter, 1995). These neuroendocrine lung tumors were comprised of invasively growing well differentiated cells that expressed the neuroendocrine markers serotonin, mammalian bombesin, calcitonin, and neuron-specific enolase, thus warranting a classification as neuroendocrine carcinomas in accordance with a recently established histological tumor classification for rodents (Nikitin, Alcaraz, Anver, Bronson, Cardiff, Dixon, Fraire, Gabrielson, Gunning, Haines, Kaufman, Linnoila, Maronpot, Rabson, Reddick, Rehm, Rozengurt, Schuller, Shmidt, Travis, Ward and Jacks, 2004). Similar to SCLC, the neuroendocrine lung tumors in hamsters did not harbor activating point mutations in k-ras while overexpressing c-myc (Miller, Baxter, Moore and Schuller, 1994). In analogy to our in vitro experiments (Jull, Plummer and Schuller, 2001), an inhibitor of PKC completely blocked the development of DEN-induced neuroendocrine lung carcinomas in hamsters with pulmonary fibrosis (Schuller, Correa, Orloff and Reznik, 1990). In addition, green tea, which contains the phosphodiesterase inhibitors caffeine and theophylline, as well as injected theophylline significantly inhibited the development of NNK-induced neuroendocrine lung carcinomas in hamsters with pulmonary fibrosis (Schuller, Porter, Riechert, Walker and Schmoyer, 2004). In conjunction with the in vitro findings described above, the data generated in these animal experiments suggest upregulation and sensitization of the $\alpha 7$ nAChR by the relatively hypoxic microenvironment in the diseased lung as an important factor in the etiology of smoking-associated SCLC. This interpretation is consistent with findings reported by Dr. Cooke during this symposium, which described upregulation of the $\alpha 7$ nAChR in endothelial cells by hypoxia (Heeschen, Weis, Aicher, Dimmeler and Cooke, 2002). Moreover, these data suggest that $\alpha 7$ nAChR antagonists, or inhibitors of PKC, Raf, or serotonin uptake may be promising agents for the prevention and therapy of SCLC.

Studies in monkeys (Sekhon, Jia, Raab, Kuryatov, Pankow, Whitsett, Lindstrom and Spindel, 1999) and in cell lines derived from a variety of normal lung cell types and from different histological types of lung cancer have identified nAChRs of many different subunit compositions, including the $\alpha 7$ subunit, in normal bronchial epithelial cells (Arredondo,

Chernyavsky and Grando, 2006b). In addition, expression of the $\alpha 7$ nAChR has been reported in small airway epithelial cells, in a lung squamous cell carcinoma and several but not all investigated PAC cell lines, and in a panel of diverse NSCLC tissue samples, including large cell carcinoma, squamous cell carcinoma, PAC and carcinoid (Plummer, Dhar and Schuller, 2005). Studies with bronchial epithelial cells BEP2D, which express a wide range of nAChR types, revealed that NNK and NNN both stimulated cell proliferation and malignant transformation, effects primarily mediated via $\alpha 7$ nAChRs by NNK and via $\alpha 3$ nAChRs by NNN (Arredondo, Chernyavsky and Grando, 2006). These studies also confirmed the earlier reported (Schuller and Orloff, 1998) very high affinities of NNK for the $\alpha 7$ nAChR and of NNN to epibatidine-sensitive nAChRs in radio-ligand binding assays. Nicotine and NNK-induced attenuation of apoptosis via activation of the serine/threonine kinase, AKT (West, Brognard, Clark, Linnoila, Yang, Swain, Harris, Belinsky and Dennis, 2003) as well as activation of NF κ B associated with upregulation of cyclin D1 (Ho, Chen, Wang, Pestell, Albanese, Chen, Chang, Jeng, Lin, Liang, Tseng, Lee, Lin, Chu, Chen, Lee, Tso, Lai and Wu, 2005) are additional $\alpha 7$ nAChR-mediated signaling events that have been reported in immortalized human bronchial and small airway epithelial cells. Both of these cell types express the EGFR and its associated mitogenic and anti-apoptotic signaling pathways. It remains to be elucidated if the reported $\alpha 7$ nAChR-mediated effects on NF κ B, cyclin D1 and AKT include cross-talk with the EGFR, upregulation of the EGFR and/or the nAChR-mediated release and re-uptake of EGF. It has been shown that nicotine upregulates the EGFR in an EGF-dependent manner in cervical cancer cell lines while also upregulating vascular endothelial growth factor (VEGF) in these cells (Lane, Gray, Mathur and Mathur, 2005; Mathur, Mathur and Young, 2000). These findings are complemented by a recent report that nicotine-induced stimulation of the $\alpha 7$ nAChR in endothelial cells causes the release of VEGF and transactivated the VEGF receptor (Heeschen, Weis, Aicher, Dimmeler and Cooke, 2002).

Conclusions and future directions

The data summarized in this review suggest an important role of nitrosamine-induced cellular signaling via the $\alpha 7$ nAChR in the development of smoking-associated SCLC and possibly, other histological types of lung cancer. In accord with one of the physiological roles of this receptor as a regulator for the release of neurotransmitters, hormones and growth factors, much of the observed cellular signaling in response to NNK-induced $\alpha 7$ nAChR stimulation in SCLC cells and their putative cells of origin, PNECs, appears to be caused by the release and re-uptake of serotonin. In NSCLCs and their putative cells of origin, large, and small airway epithelial cells, the reported signaling responses to NNK, nicotine, or NNN mediated by the $\alpha 7$ nAChR and epibatidine-sensitive nAChRs may involve transactivation and upregulation of the EGFR, with or without the concomitant release and re-uptake of EGF, a hypothesis warranting future studies. The very high affinities of NNK to the $\alpha 7$ nAChR and of NNN to epibatidine-sensitive nAChRs strongly suggest that these two nitrosamines play important roles in a host of biological effects that have to this date been attributed to nicotine.

An important aspect of nAChR biology is the fact that the functions of these receptors are cell type-specific and that their expression levels and sensitivity can be modulated by a host of factors in the human environment. Exposure to nicotine or NNK by smoking or second-hand smoke upregulates the $\alpha 7$ nAChR, as does a hypoxic environment. Both of these conditions are met in smokers who develop chronic non-neoplastic pulmonary diseases prior to the onset of lung cancer. Pending on their medical history, there will therefore be significant inter-individual variations in the levels of expression and sensitivity of this receptor in identical types of cells. Similar to other nicotinic receptor ligands, NNK also binds as a high affinity agonist to β -adrenoreceptors (β -ARs) and has been shown to stimulate the proliferation of small airway epithelial cells and the adenocarcinomas derived from them via PKA-dependent transactivation of the EGFR (Laag, Majidi, Cekanova, Masi, Takahashi and Schuller, 2006; Schuller, 2002).

Again, the expression and sensitivity of these receptors are greatly influenced by exposure to agonists (downregulation) widely used for the treatment of asthma, antagonists (upregulation), widely used as anti-hypertensive agents as well as agents that increase intracellular cAMP, such as caffeine and theophylline (sensitization). Whether or not cellular responses to NNK are mediated by nAChR or β -AR signaling will thus vary considerably from one individual to the next. This heterogeneity of nicotinic and beta-adrenergic receptor expression and sensitivity is still maintained in cell lines derived from human patients, a fact often overlooked by the scientific community. In order to arrive at the design of effective lung cancer prevention and treatment strategies, diagnostic tools for the identification of hyperactive signaling pathways in the individual patient are urgently needed.

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Figure 1. Structure of nitrosamines. Nitrosamines are formed from the reaction of amines with nitrosating agents and are characterized by the presence of the nitroso-group (N-NO). Pending on the structure of the amine involved in their formation, nitrosamines may may be simple, aliphatic (A) or ring structures (B).

Acetylcholine **N-Nitrosodiethylamine
(DEN)** **Nicotine** **N-Nitrososornicotine
(NNN)** **4-Methylnitrosamino-
1-(3-pyridyl)-1-butanone
(NNK)****Figure 2.**

Structures of the physiological agonist for nAChRs, acetylcholine, the tobacco alkaloid, nicotine, the aliphatic nitrosamine, DEN, and the two nicotine-derived nitrosamines, NNN and NNK.

Figure 3.

A. Results of radioligand binding assays in the human SCLC cell line, NCI-H69. Nicotine, NNK, NNN, and DEN competed with [125 I] α BTX (20 nM) for α 7nAChR bindings sites. Analysis of the displacement data (triplicate samples per data point from two replicate experiments) was by nonlinear regression for a single class of receptors.

B. Results of radioligand binding assays in the human PAC cell line, NCI-H441. Nicotine, NNK, NNN, and DEN competed with [3 H]epibatidine (20 nM) for epibatidine-sensitive nAChR bindings sites. Analysis of the displacement data (triplicate samples per data point from two replicate experiments) was by nonlinear regression for a single class of receptors.

Figure 4.
Simplified scheme illustrating the cooperative regulation of SCLC cells and PNECs by the $\alpha 7$ nAChR and the hypoxia receptor.