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Mechanisms of growth-promoting and tumor-protecting effects of epithelial nicotinic acetylcholine receptors

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ABSTRACT

Although the role of nicotine as a carcinogen is debatable, it is widely accepted that it contributes to cancer by promoting growth and survival of mutated cell clones and protecting them from the chemo- and radiotherapy-induced apoptosis. On the cell membrane (cm), the nicotinic acetylcholine (ACh) receptors (nAChRs) implement upregulation of proliferative and survival genes. Nicotine also can permeate cells and activate mitochondrial (mt)-nAChRs coupled to inhibition of the mitochondrial permeability transition pore (mPTP) opening, thus preventing apoptosis. In this study, we sought to pin down principal mechanisms mediating the tumor-promoting activities of nicotine resulting from activation of cm- and mt-nAChRs in oral and lung cancer cells, SCC25 and SW900, respectively. Activated cm-nAChRs were found to form complexes with receptors for EGF and VEGEF via the α7 and β2 nAChR subunits, respectively, whereas activated mt-nAChRs physically associated with the intramitochondrial protein kinases PI3K and Src via the α7 and β4 subunits. This was associated with upregulated expression of cyclin D1/activation of ERK1/2 and inhibition of mPTP opening, respectively, as well as upregulated proliferation and resistance to H_2O_2-induced apoptosis. The molecular synergy between cm-nAChRs and growth factor receptors helps explain how one biological mediator, such as ACh, can modulate activity of the other, such as a growth factor, and *vice versa*. Establishment of functional coupling of mt-nAChRs to regulation of mPTP opening provides a novel mechanism of nicotine-dependent protection from cell death. Further elucidation of this novel mechanism of tumor-promoting activities of nicotine should have a strong translational impact, because extraneuronal nAChRs may provide a novel molecular target to prevent, reverse, or retard progression of both nicotine-related and unrelated cancers.

1. Introduction

Electronic cigarettes (**eCigs**) are rapidly gaining acceptance as an alternative to conventional cigarettes source of nicotine with little knowledge regarding their biomedical effects [1]. Both oral cavity and airways are exposed to nicotine in eCig vapers. Nicotine contributes to cancer mainly by promoting growth and survival of mutated cell clones and protecting them from the chemo- and radiotherapy-induced apoptosis (reviewed in [2]). In so doing, nicotine implements a "second hit" that fosters survival and expansion of cells with the genomic damage induced by "professional" carcinogens (an epigenetic effect [3]). On the other hand, inactivation of nicotinic acetylcholine (**ACh**) receptors (**nAChRs**) can attenuate nicotine- or tobacco nitrosamine-induced cell proliferation [4–12], and suppress nicotine-dependent chemoresistance [13]. Therefore, nAChRs are viewed as a novel drug target for prevention and treatment of various forms of cancers [2].

Current research indicates that the tumor-promoting activities of nicotine are mediated by extraneuronal nAChRs. Nicotine can displace ACh from nAChRs due to the higher receptor-binding affinity [14,15], and also can upregulate nAChR expression [16]. The nAChRs are classic representatives of superfamily of the ligand-gated ion channel pentameric receptor proteins composed of ACh binding α subunits and "structural" subunits [2]. Oral keratinocytes and airway epithelial cells can express the α3, α5, α7, α9, α10, β2 and β4 nAChR subunits [17–22]. The differences in subunit composition determine the functional characteristics of the receptor formed, so that the net biological effect produced by a nicotinic agonist depends on the subtype of nAChR binding this ligand with the highest affinity. It has been reported that nAChR subunit proteins can physically associate with both protein kinases and protein tyrosine phosphatases in large

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multimeric complexes [23–29], and that activation of nAChR by nicotine involves phosphorylation and activation of epidermal growth factor (**EGF**) receptor (**EGFR**) [30,31]. We were first to demonstrate that stimulation of keratinocyte nAChRs triggers protein kinase signaling cascades, thus simultaneously altering gene expression and inducing reciprocal changes in the cytoskeleton [32,33].

It is well-documented that cell membrane (**cm**)-nAChRs can mediate the nicotine-dependent upregulation of proliferative and survival genes, thus contributing to the growth and progression of cancer cells *in vitro* and *in vivo* [34–41]. Nicotine also can permeate epithelial cells and activate the mitochondrial (**mt**)-nAChRs that have been recently found on the mitochondrial outer membrane, wherein they can facilitate cell survival by inhibiting intrinsic apoptosis [42–48].

Demonstration of the mt-nAChRs preventing mitochondrial permeability transition pore (**mPTP**) opening was in keeping with independent reports about both the presence of nAChRs on mitochondria [49] and the mitochondria-protecting effects of nicotine [50,51]. In a recent study, we established the ligand-binding abilities of mt-nAChRs and demonstrated that quantity of the mt-nAChRs coupled to inhibition of mPTP opening increases upon malignant transformation [48]. In the cytosol, nicotine can shift the dynamic equilibrium of the physiological regulation of cell survival and death, because it is insensitive to the regulatory action of intracellular acetylcholinesterase (**AChE**) that hydrolyzes ACh in the cytoplasm [52] and thus exerts the physiological control of anti-apoptotic action of mt-nAChRs, similar to the effect of the cell membrane-anchored AChE hydrolyzing extracellular ACh.

Increasingly, a wider role for ACh in various aspects of non-neuronal cell biology is being recognized, including proliferation, differentiation, apoptosis, adhesion and motility (reviewed in [53,54]). ACh is produced practically by all types of human cells, and is remarkably abundant in surface epithelia [55–60]. The concentration of ACh is a function of its synthesis by choline acetyltransferase and hydrolysis by AChE, both of which are present in the oral and airway epithelia and cultured oral and bronchial cells [17,60–62]. The extracellular pool of ACh is replenished by secretion of the ACh-containing vesicles, whereas the intracellular pool is represented mainly by free cytoplasmic ACh [63,64]. The causative role of activation of cm-nAChRs, rather than mt-nAChRs, in the growth-promoting action of nicotine was illustrated by the ability to abolish nicotine effects using the cell membrane-impermeable nAChR antagonists [65,66]. The emerging picture is that a diversity of signaling circuitries regulating cancer cell growth signifies cross-talk interactions between cm-nAChRs and growth factor (**GF**) receptors (**GFRs**), and receptors to various other autocrine and paracrine mediators [2]. We have recently demonstrated that the growth-promoting effect of nicotine mediated by activation of α7 cm-nAChR synergizes mainly with that of EGF and α3 — with vascular endothelial GF (**VEGF**) [48]. Thus, the cumulative results obtained by us and other workers suggested that that the biological sum of simultaneous activation of cm- and mt-nAChRs produces a combination of growth-promoting and anti-apoptotic signals that implement the tumor-promoting action of nicotine.

The major goal of this study was to pin down the principal molecular mechanisms mediating the tumor-promoting actions of nicotine resulting from activation of cm- and mt-nAChRs in oral and lung cancer cells. We demonstrated that growth promoting effects of nicotine on oral and lung cancer cells can result from the synergistic signaling downstream of activated cm-nAChRs forming complexes with receptors to GFs, such as EGF and VEGF. In turn, activation of mt-nAChRs inhibited an apoptogen-induced mPTP opening, which was associated with formation of complexes between mt-nAChR subunits and intramitochondrial kinases, such as PI3K and Src. Both α7 and non-α7 nAChR subtypes were involved in the growth-promoting and tumor-protecting effects of nicotine. Further elucidation of this novel mechanism of tumor-promoting activities of nicotine should have a strong translational impact.

2. Materials and methods

2.1. Cells and reagents

The oral squamous cell carcinoma cell line SCC25 and the tumorigenic line of grade IV lung squamous cell carcinoma SW900 were purchased from ATCC (Manassas, VA) and propagated in the ATCC-formulated specialized media. The nAChR agonist nicotine, as well as α-bungarotoxin (**αBtx**) – the preferring specific inhibitor of α7 nAChR [67] that also can block α9-made nAChRs [68], mecamylamine (**Mec**) – a preferential blocker of the "ganglionic" nAChR subtypes, such as α3-made nAChRs [69], the metabolic inhibitor of ACh synthesis hemicholinium-3 (**HC-3**), which inhibits ACh synthesis by blocking cellular reuptake of its metabolic precursor choline [70], staurosporine, heat-inactivated newborn calf serum and all secondary antibodies were purchased from Sigma-Aldrich Corporation, Inc. (St. Louis, MO). Antibodies to EGFR (ErbB1 or HER1), and VEGF receptor-2 (**VEGFR2**) both were from Thermo Fisher Scientific (Rockford, IL). Anti-cyclin D1 antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX), anti-PI3K p85 antibody – from Antibodies-online Inc. (Atlanta, GA) and anti-Src antibody – from Proteintech Group, Inc. (Chicago, IL). The human recombinant EGF activating EGFR [71] was obtained from R&D Systems, Inc. (Minneapolis, MN) and VEGF, which binds to both VEGFR1 and VEGFR2 [72] — from Abcam (Cambridge, MA). The phospho-ERK1/2 (Thr185/Tyr187) ELISA kit was purchased from Millipore (Billerica, MA) and used to determine the activity of this kinase following the protocol provided by the manufacturers, as we have reported elsewhere [33].

2.2. Cell viability, proliferation and apoptosis assay

The SCC25 and SW900 cells were seeded at a density of 1×10^4 per well of a 96-well plate and incubated for 24 h in the absence (intact control) or presence of 0.1 μM nicotine ± 10 μM Mec and/or 1 μM αBtx or 10 ng/ml of each tested GF, after which the cells were suspended and the numbers of the trypan blue dye (**TBD**)-positive (dead) and -negative (alive) cells were measured. The test cells in suspension were mixed with 0.4% TBD (Sigma-Aldrich Corporation) in PBS, and then visually examined in a hemocytometer to determine the number of viable cells with clear cytoplasm and non-viable cells with a blue cytoplasm, as detailed elsewhere [73]. To induce programmed cell death, we used an established model of reactive oxygen species-mediated apoptosis [74]. The control and experimental cell monolayers were incubated with 100 μM H_2O_2 in culture medium for 12 h, after which the cells undergoing apoptosis were visualized using the DeadEnd™ Fluorometric TUNEL (i.e., the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling) System purchased from Promega (Madison, WI).

2.3. Quantitative (q)PCR assay

Total RNA was extracted from SCC25 or SW900 cells at the end of exposure experiments with the RNeasy Mini Kit (Qiagen, Valencia, CA) and used in the qPCR assays of cyclin D1 gene expression. The qPCR primers were designed with the assistance of the Primer Express software version 2.0 computer program (Applied Biosystems, Foster City, CA) and the service Assays-on-Demand provided by Applied Biosystems. The qPCR reactions were performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) and the TaqMan Universal Master Mix reagent (Applied Biosystems) in accordance to the manufacturer's protocol, as detailed by us elsewhere [32]. To correct for minor variations in mRNA extraction and reverse transcription, the gene expression values were normalized using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The data from triplicate samples were analyzed with a sequence detector software (Applied

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