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The nicotinic acetylcholine receptor-mediated reciprocal effects of the tobacco nitrosamine NNK and SLURP-1 on human mammary epithelial cells

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ABSTRACT

Recent research has demonstrated that the nicotinergic signaling network of mammary epithelium can both mediate the physiological control of normal breast epithelial cells (BECs) and exhibit tumor-promoting effects on malignant BECs. Therefore, mammary nicotinic acetylcholine (ACh) receptors (nAChRs) may become a specific target for novel anti-breast cancer therapies. Toward this goal, we investigated the difference in the ACh receptor repertoires between normal and malignant BECs, determined effects of nicotinic ligands on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-dependent activation of ERK1/2 and tumorigenic transformation of MCF10A cells, and characterized reciprocal effects of NNK and SLURP (secreted mammalian Ly-6/urokinase plasminogen activator receptor related protein-1)-1 on the expression of nAChR subunits and several oncogenes and tumor-suppressing genes in BECs. Both the non-malignant MCF10A and malignant MCF7 breast cells expressed α3, α5, α7, α9, α10, β1, β2, γ, δ and ε nAChR subunits and M_1, M_3, M_4 and M_5 muscarinic receptor subtypes. The malignancy was associated with expression of α1, α4 and β4 nAChR subunits and M_2 subtype. Malignant transformation of BECs was also associated with overexpression of α7-, and α9-made nAChRs. NNK upregulated ERK1/2 phosphorylation, stimulated expression of the gene encoding the tumor-promoter HGF, downregulated expression of the tumor suppressor gene CDKN2A, and induced tumorigenic transformation of MCF10A cells. Compared to the canonical nAChR antagonists, SLURP-1 showed the highest ability to abolish the nAChR-mediated effects of NNK in both cell-signaling and cell-transformation assays and reversed many effects of NNK on gene expression. SLURP-1 also markedly upregulated the tumor suppressor genes CDKN2B, RUNX3 and TP73. Altogether, the obtained results provided new insight into the molecular mechanisms of nAChR-mediated oncogenic effects of NNK on BECs and demonstrated the ability to abolish or reverse these effects by SLURP-1.

1. Introduction

Development of new therapeutics for breast cancer (**BC**) is one of the overarching challenges of contemporary medicine, because currently used interventions do not change the mortality statistics, and the cost of treating BC continues to rise [1–4]. A novel, paradigm-shifting approach is suggested by recent discoveries in the mechanisms of BC development, placing mammary nicotinic acetylcholine (**ACh**) receptors (**nAChRs**) in the pathophysiological loop [5]. In addition to its well-known function as a cholinergic neurotransmitter, ACh has emerged as a candidate for a regulatory role in numerous biological processes that are intimately connected to each other, including proliferation, differentiation, apoptosis, adhesion and migration of non-neuronal cells (reviewed in [6,7]). ACh is produced by practically all types of live cells, including normal and malignant breast epithelial cells (**BECs**) [5,8]. Although nAChR is an ion channel mediating influx of Na^+ and Ca^{2+} and efflux of K^+, its activation elicits both ionic and non-ionic signaling events regulating phosphorylation and dephosphorylation of target proteins (reviewed in [9]). The nAChR subunit proteins can physically associate with both protein kinases and protein tyrosine phosphatases in large multimeric complexes [10]. Even a short-term exposure to nicotine activates mitogenic signaling pathways involving signaling kinases [11]. We were first to demonstrate that stimulation of nAChRs concurrently triggers several protein kinase signaling cascades, thereby simultaneously altering gene expression and inducing reciprocal cytoskeleton changes [12].

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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, i.e., α1–α10, β1–β4, γ, δ and ε subunits (reviewed in [9]). Each of α7, α8 (not found in humans) and α9 subunits is capable of forming the homomeric nAChR channels. The heteromeric receptors can be composed of various combinations of α1, α2, α3, α4, α5, α6, β1, β2, β3 and β4 subunits, e.g., α3(β2/β4) ± α5, and α9 can form a heteromeric receptor with α10. The differences in subunit composition determine the functional and ligand-binding characteristics of the nAChR 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. The nicotinergic agonist nicotine binds to and activates nAChRs with a higher affinity than ACh, stimulating growth and suppressing apoptosis of cancer cells, thereby acting as a tumor promoter [9]. Therefore, not surprisingly, nicotine has been found to alter expression of a large number of genes involved in cellular and metabolic processes of BECs, including the genes linked to cancer [13].

There is sufficient evidence to hypothesize that the nicotinergic signaling network of mammary epithelium can both mediate the physiological control of normal BECs and exhibit tumor-promoting effects on malignant BECs. It has been demonstrated that stimulation of the nicotinic arm of ACh signaling increases proliferation rate and estrogen receptor expression in normal human BECs [14]. nAChR activation also favors propagation of mutated cell clones, which contributes to tumor progression and metastases, and interferes with anti-cancer therapies aimed at the induction of BC apoptosis (reviewed in [5]). An unopposed cholinergic stimulation due to inhibition of the ACh degrading enzyme acetylcholinesterase by the organophosphorous pesticides parathion and malathion leads to BC formation in rats [15]. These environmental toxins are believed to play a role in the etiology of BC [16]. Furthermore, it has been reported that smoking increases risk of BC initiation and recurrence and a poor prognosis among women diagnosed with BC and a higher BC mortality [17–19]. Recent studies have demonstrated that BC progression is associated with overexpression of α9 nAChR [20], which may allow cancer cells to capture higher amounts of ACh to facilitate their rapid growth. It is worthy of note that polymorphisms of α9 nAChR gene and smoking exposure synergize to increase the risk of BC [21]. It has been also documented that α7 nAChR, which is known to be overexpressed in lung cancer [22], is involved in the proliferation of the BC stem cells that constitute a minor subpopulation responsible for tumor development and metastasis [23]. Most importantly, it has been recently reported that activation of nAChRs can both inhibit apoptosis of BC cells and engage epidermal growth factor (**EGF**) receptor-coupled signaling pathways to stimulate BC progression [24,25]. On the other hand, inhibition of signaling through mammary nAChRs suppresses nicotine-dependent proliferation of BC cells [26] and produces anti-tumor effects [27,28]. Therefore, mammary nAChRs may become a specific target for novel anti-cancer therapies [20,22].

Recent progress made in elucidation of the biological and clinical significance of non-canonical ligands of nAChRs has indicated that both α7 and non-α7 nAChRs can mediate pathobiologic effects of tobacco nitrosamines, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (**NNK**), on epithelial cells (reviewed in [29]). Exposure of the human non-malignant BEC line MCF10A to NNK results in cellular carcinogenesis [30,31]. We have demonstrated that while NNK binds to nAChRs with a higher than ACh affinity, and evokes an agonistic response upregulating cell growth, nicotinic antagonists can abolish pathobiological effects of NNK [32,33]. Thus, it appears that in addition to a well-formulated role of genotoxic damage in the etiology of tobacco-related cancer, *N*-nitrosamines may contribute directly to tumorigenesis through the nAChR-mediated mechanisms. We have also demonstrated that SLURP (**s**ecreted mammalian **L**y-6/**u**rokinase plasminogen activator **r**eceptor related **p**rotein-1)-1 and -2, autocrine and paracrine ligands of epithelial nAChRs, can alleviate tumorigenic effects of NNK [34], suggesting that SLURPs may be useful for pharmacological control of the NNK-dependent alterations of BECs.

In this study, we identified the difference in the ACh receptor repertoires between normal and malignant BECs, determined effects of canonical and non-canonical ligands of nAChRs on NNK-dependent activation of ERK1/2 and tumorigenic transformation of MCF10A cells, and characterized reciprocal effects of NNK and SLURP-1 on the expression of α7 and α9 genes as well as several oncogenes and tumor-suppressing genes in BECs. Altogether, the obtained results provided new insight into the molecular mechanisms of nAChR-mediated oncogenic effects of NNK on BECs and suggested new approaches to prevention and treatment of BC.

2. Materials and methods

2.1. Cells and reagents

The spontaneously immortalized, non-tumorigenic human BEC line MCF10A [35] and the human breast adenocarcinoma cell line MCF7 were from American Type Culture Collection (Manassas, VA). MCF10A cells were grown in growth media consisted of DMEM/F12, supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, and 10 μg/ml insulin. MCF7 cells were grown in DMEM supplemented with 10% fetal bovine serum. In the focus-formation assay, the cells were grown in T-25 flasks for 14 days by changing medium every 3 days, washed and stained by 0.05% crystal violet in 80% methanol [36]. The foci were counted in three 1 cm^2 randomly selected fields in each flask. The carcinogenic tobacco nitrosamine NNK was purchased from Toronto Research Chemicals (North York, ON, Canada). The nicotinic antagonists mecamylamine (**Mec**) — a preferential blocker of heteromeric nAChR subtypes, such as α3- or α4-made nAChRs, and the specific inhibitors of the homomeric nAChR subtypes, such as α7 and α9, α-bungarotoxin (**αBtx**) and methyllycaconitine (**MLA**) [37] were from Sigma-Aldrich Corporation (St Louis, MO). The full length recombinant SLURP-1 and SLURP-2 proteins were manufactured at Virusys Corporation (Sykesville, MD), as detailed by us before [38]. Rabbit monoclonal anti-pERK1/2 and anti-ERK1/2 and secondary goat anti-rabbit-HRP antibodies were from Cell Signaling Technology (Beverly, MA, USA).

2.2. PCR experiments

A standard reverse-transcription PCR (**RT-PCR**) assay was performed using primers for human nAChR subunits and muscarinic ACh receptor (**mAChR)** subtypes and reaction conditions described by us elsewhere [39]. For quantitative real-time (**q**)PCR, we used primers for α7 (cgcaaccactcaccgtctacttct and cacccctggatattctgacacatt) and α9 (aagtgctgacgaggaata and tgtgactaatccgctctt) nAChR subunits and reaction conditions described by us in the past [40]. The PCR array experiments were performed using the RT^2-Profiler PCR Array for Human Oncogenes & Tumor Suppressor genes (PAHS-502E, SABiosciences) and ABI Prism 7900 HT (Applied Biosystems, Carlsbad, CA), as detailed by us elsewhere [41]. Briefly, total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA). The First-Strand Kit, which contains a mixture of oligo(dT) primer and random hexamers (SABiosciences, Frederick, MD), was utilized for cDNA synthesis, using 400 ng of total RNA from experimental and control (untreated) cells. Analyses of the raw data were done through the SuperArray Data Analysis Web Portal (SABiosciences).

2.3. ERK1/2 phosphorylation studies

At approximately 60% confluence, MCF10A cells were exposed NNK ± test nAChR ligands, incubated for the specified periods of time (antagonists/inhibitors were preincubated with cells for 1 h), washed twice with ice-cold PBS, and scraped into a buffer containing 120 mM NaCl, 1% Triton X-100, 40 mM HEPES (pH 7.4), 1 mM Na_2EDTA, 10 mM Na pyrophosphate, 10 mM Na glycerophosphate, 50 mM NaF,

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