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Receptor-mediated tobacco toxicity:

Alterations of the NF- κ B expression and activity downstream of α 7 nicotinic receptor in oral keratinocytes

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Abstract

To gain a mechanistic insight into nicotinic receptor-dependent morbidity of tobacco products in the oral cavity, we studied effects of exposures of normal human oral keratinocytes (KCs) for 24 h to environmental tobacco smoke (ETS) vs. equivalent concentration of pure nicotine. The exposed KCs showed a multifold increase of nuclear factor- κ B (NF- κ B) at the mRNA and protein levels, which could be significantly ($p < 0.05$) diminished by α -bungarotoxin or transfection with anti- α 7 small interfering RNA. An increased protein-binding activity of NF- κ B also could be prevented by blocking α 7 signaling. The use of pathway inhibitors demonstrated that the Ras/Raf-1/MEK1/ERK steps mediated α 7-dependent upregulation of NF- κ B. Thus, exposure of KCs to tobacco may lead to the pathobiologic effects *via* an intracellular signaling pathway downstream of α 7 that proceeds through the Ras/Raf-1/MEK1/ERK steps leading to upregulated expression and transactivation of NF- κ B.

Keywords

nicotinic acetylcholine receptors; gene expression; NF- κ B; Ras; Raf; ERK; MEK

INTRODUCTION

Previous studies identified α 7 nicotinic acetylcholine receptor (nAChR) as the principal receptor subtype mediating effects of tobacco products and pure nicotine on epithelial cells (Cattaneo et al., 1997; Codignola et al., 1994; Schuller, 1989; Schuller et al., 2000; SchullerOrloff, 1998; Schuller et al., 2003). We have demonstrated that α 7 nAChR is essential for a sustained turnover of the mucocutaneous epithelium in humans (Arredondo et al., 2002). The α 7 nAChR is also expressed in human cancer cells, and its activation accelerates tumors progression, suggesting that the α 7 nAChR-coupled signaling may play an important role in the development of tobacco-related cancers (Ehrhardt et al., 2003; Plummer et al., 2005; Ye et al., 2004). The α 7 subunit is first expressed on the cell surfaces of oral keratinocytes (KCs) comprising the lower third portion of the gingival epithelium, and becomes abundant at

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the terminal stage of cell development in the gingival epithelium (Nguyen et al., 2000). In a recent study, we found that the $\alpha 7$ inhibitor α -bungarotoxin (α Btx) and transfection with the small interfering RNA against $\alpha 7$ (siRNA- $\alpha 7$) can block effects of environmental tobacco smoke (ETS) and pure nicotine on human KCs (Arredondo et al., 2006b; Chernyavsky et al., 2005). The signaling through the Ras/Raf-1/MEK1/ERK steps mediated, in the most part, the $\alpha 7$ -dependent effects. Targeted mutation of the $\alpha 7$ gene prevented ERK1/2 activation by nicotine. Using the electrophoretic mobility shift assay (EMSA), we have also demonstrated that an increased protein-binding activity of STAT-3 caused by ETS/nicotine was mediated by JAK-2 (Arredondo et al., 2006b).

To further define the pathways mediating $\alpha 7$ signaling in KCs exposed to tobacco products, we in the present study investigated the role for the signal transduction effector nuclear factor- κ B (NF- κ B). Both environmental tobacco smoke and smokeless tobacco exhibit some of their pathobiologic effects at the cellular level through transactivation of NF- κ B (Anto et al., 2002; Manna et al., 2006; Petro, 2003; Shen et al., 1996). The involvement of NF- κ B in nicotine effects have been described in various types of normal and malignant cells (Cirillo et al., 2006; Ho et al., 2005; Lau et al., 2006; Manna et al., 2006; Tsurutani et al., 2005). It has been shown that stimulation of cells via nAChRs results in NF- κ B activation (Heeschen et al., 2002), and that Ras/Raf-1/ERK pathway can mediate NF- κ B activation (Chen et al., 2004).

We found that in normal human oral KCs, activation of $\alpha 7$ nAChR leads to upregulated gene expression and transcriptional activity of NF- κ B, which may play a role in mediating the pathobiologic effects of tobacco products in the mucocutaneous epithelium.

MATERIALS AND METHODS

Reagents

α Btx and nicotine were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). The noncompetitive inhibitor of the Ras acceptor protein manumycin A, the cRaf-1 kinase inhibitor GW5074, the MEK inhibitor I and U0126 as well as the negative control U0124 were from Calbiochem-Novabiochem Corp. (La Jolla, CA). The plasmids encoding the constitutively active MEK1 (CA-MEK), the dominant negative MEK1 mutant (DN-MEK) and the wild-type control MEK1 were purchased from Biomx Technology (San Diego, CA). The siRNA- $\alpha 7$ and negative control siRNA were from Dharmacon (Lafayette, CO).

Normal human oral KCs

The KCs were obtained from attached gingiva as detailed elsewhere (Nguyen et al., 2000) and grown at 37°C and 5% CO₂ in serum-free KC growth medium containing 0.09 mM Ca²⁺ (KGM; Gibco BRL, Gaithersburg, MD). KCs were transfected with siRNAs and MEK1 kinase mutants following a standard protocol (Chernyavsky et al., 2005). The monolayers grown to ~80% confluence in 6-well plates were incubated for 24 h in KGM pre-exposed to ETS in the chambers of a sidestream smoke exposure system, as detailed elsewhere (Arredondo et al., 2006b), or containing an equivalent concentration of nicotine (10 μ M), and used in the biochemical assays described below.

Biochemical assays

In each individual culture, 2.5×10^6 viable KCs were used to extract total RNA and proteins. The real-time PCR, the in-cell western assay (LI-COR Lincoln, NE) and EMSA were performed as described in detail elsewhere (Arredondo et al., 2006b). For real-time PCR, the primers of the gene encoding human NF- κ B 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-Design provided by Applied Biosystems. Obtained gene expression

values were normalized using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. For EMSA, the oligonucleotide 5'-AGT TGA GGG GAC TTT CCC AGG C-3' supplied by the Operon (Alameda, CA) was labeled with digoxigenin and its complement. All experiments were performed in triplicates and the results were expressed as mean $\pm$ SD. Statistical significance was determined using Student's *t*-test.

RESULTS

$\alpha 7$ nAChR mediates the effects of ETS and nicotine on expression of NF- κ B in KCs through the Ras/Raf-1/MEK1/ERK pathway

By both real-time PCR and in-cell western, ETS and pure nicotine induced multifold increases of NF- κ B in KCs (Fig. 1). These effects could be significantly ($p < 0.05$) diminished if the cells were pretreated with α BTX or transfected with siRNA- $\alpha 7$. The elevation of NF- κ B could be also alleviated by the Ras inhibitor manumycin A, and the cRaf-1 inhibitor GW5074, which, however, did not affect the upregulation caused by transfection of KCs with CA-MEK (Fig. 1). The MEK inhibitors blocked upregulated expression of NF- κ B, indicating to an upstream involvement of the MEK1/ERK steps. As expected, DN-MEK, but not wild-type control MEK, inhibited the effects of ETS/nicotine (Fig. 1). Co-transfection of KCs with control MEK and siRNA- $\alpha 7$ also abolished ETS/nicotine-dependent NF- κ B upregulation (Fig. 1).

Activation of the transcriptional activity of NF- κ B in response to $\alpha 7$ nAChR stimulation

The EMSA demonstrated that exposure of KCs to either ETS or nicotine in both cases increase the protein-binding activity of NF- κ B (Fig. 2). The role for $\alpha 7$ -dependent signaling was investigated by pretreating the KCs with α BTX or transfecting them with siRNA- $\alpha 7$. In both cases, the upregulated activity of NF- κ B could be abolished (Fig. 2).

DISCUSSION

The results of the present study demonstrated that in human oral KCs, activation of $\alpha 7$ nAChR can mediate stimulatory effects of tobacco products on the gene expression and transcriptional activity of NF- κ B. The intracellular signaling downstream of $\alpha 7$ leading to upregulation of NF- κ B proceeded through the Ras/Raf-1/MEK1/ERK steps. Previous studies identified $\alpha 7$ is one of the major acetylcholine-gated ion channels on the KC cell membrane that mediates acute effects of tobacco products on oral KCs (Arredondo et al., 2006b), whereas the effects of long-term exposures (lasting 5 days) are predominantly mediated by the $\alpha 3$ -made nAChR channels, such as $\alpha 3\beta 2$ (Arredondo et al., 2005). Interestingly, NF- κ B is targeted by both the $\alpha 7$ - and the $\alpha 3$ -coupled pathways. Since KC exposures to tobacco products and nicotine can modify the repertoire of nAChRs expressed by the cells, the identification of nAChR subtype-selective control of the gene expression may provide a mechanistic insight into the deleterious effects of tobacco and nicotine in the mucocutaneous epithelium.

Activation of $\alpha 7$ nAChRs by the nicotine-derived nitrosamines has been shown to lead to malignant transformation of oral cells (Arredondo et al., 2006a). Just like normal KCs used in this study, the immortalized Het-1A cells stimulated with nitrosamines showed upregulated expression of NF- κ B. The role for NF- κ B in mediating nicotine-dependent decrease of apoptosis has been demonstrated in lung cancer cells (Tsurutani et al., 2005). Nitrosamines stimulate normal human bronchial cell proliferation through activation of the NF- κ B (Ho et al., 2005), and promote colon cancer growth in vitro via NF- κ B that conveys the biological effect of $\alpha 7$ -nAChR (Ye et al., 2004). Therefore, transcriptional and posttranscriptional activation of this signal transduction effector in oral KCs may play a key role in the pathogenesis of head and neck cancer by acting as tumor promoters that facilitate the outgrowth of cells with genetic damage.

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Fig 1. Alterations in NF- κ B gene expression at the mRNA and protein levels in human KCs exposed to ETS or pure nicotine

The real time-PCR (A,C) and in-cell western (B,D) analyses of ETS (A,B) and nicotine (C,D) effects on the NF- κ B gene expression in human KCs after 24 h incubation in KGM pre-exposed to ETS or containing 10 μ M nicotine. The gene expression ratios in the control cells, i.e., intact KCs and KCs transfected with a control siRNA in experiments with siRNA- α 7, were taken as 1. The following experimental treatments were used: 3 μ M manumycin A (Mnmc); 3 μ M manumycin A on KCs transfected with CA-MEK (CA-MEK+Mnmc); 0.1 μ M GW5074; 0.1 μ M GW5074 on the CA-MEK transfected KCs (CA-MEK+GW5074); 1 μ M MEK inhibitor I (MEK-Inh); 10 μ M U0126; transfection with DN-MEK; transfection with the control, wild-

type MEK mutant (WT-MEK); 1 μ M α Btx; transfection with siRNA- α 7; and co-transfection with siRNA- α 7 and CA-MEK (CA-MEK+siRNA- α 7), WT-MEK (WT-MEK+siRNA- α 7) or DN-MEK (DN-MEK+siRNA- α 7). Asterisks indicate significant ($p<0.05$) differences from intact control KCs. The pound signs indicate significant ($p<0.05$) differences from KCs exposed to either ETS or nicotine alone.

Fig 2. Activation on the protein-binding activity of NF-κB

The KCs were treated with ETS or pure nicotine either in the presence of 1 μ M α BTX or being transfected with siRNA- α 7, and then subjected to functional analysis of NF-κB. The EMSA was used to detect the DNA-protein complex in the nuclear extracts prepared from experimental and control KCs, as detailed in Material and Methods. *Lane 1*: nicotine; *lane 2*: ETS; *lane 3*: nicotine + α BTX; *lane 4*: ETS + α BTX; *lane 5*: nicotine + siRNA- α 7; *lane 6*: ETS + siRNA- α 7; *lane 7*: untreated control cells.