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Research paper

Inhibitory effects of nicotine derived from cigarette smoke on thymic stromal lymphopoietin production in epidermal keratinocytes

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ABSTRACT

Thymic stromal lymphopoietin (TSLP) is regarded as the main factor responsible for the pathogenesis of atopic dermatitis (AD). Cigarette smoke is an aggravating factor for allergies, but has been reported to decrease the risk of AD. In the present study, we evaluated the role of nicotine, the main constituent in cigarette smoke extract, and its underlying mechanism of action in the regulation of TSLP expression. We found that nicotine significantly inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced TSLP expression in BALB/c mice and the mouse keratinocyte cell line PAM212. Nicotine inhibition of TSLP production was abolished by pretreatments with α7 nicotinic acetylcholine receptor (α7 nAChR) antagonists, AMP-activated protein kinase (AMPK) inhibitor, and phosphoinositide 3-kinase (PI3K) inhibitors. The same inhibitors abolished inhibition of nuclear factor-κB (NF-κB) activation by nicotine. These results suggest that nicotine inhibits the expression of TSLP by suppressing the activation of NF-κB through the α7 nAChR–PI3K–AMPK signaling pathway.

1. Introduction

The prevalence and severity of atopic dermatitis (AD) have been increasing in recent years, and this condition is caused by allergen-induced T helper 2 (Th2) cellular responses characterized by the production of Th2 cytokines [1,2]. Large amounts of thymic stromal lymphopoietin (TSLP), an interleukin-7 (IL-7)-like cytokine, are produced by epithelial cells in AD lesions. TSLP appears to be a master regulator of Th2-type allergic inflammation by inducing the maturation, activation, and migration of dendritic cells (DCs) (Langerhans cells) and Th2-type lymphocytes [3–7]. The overexpression of TSLP in keratinocytes has been shown to trigger an AD-like skin phenotype in mice, suggesting that it is responsible for the initiation, maintenance, and aggravation of AD [8–10]. Previous studies have demonstrated that the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways play crucial roles in the expression of TSLP [11,12].

Among several indoor pollutants, cigarette smoke has been suggested as a risk factor contributing to the development of AD [13–15]. On the other hand, exposure to cigarette smoke during early adult life as a result of parental smoking or personal smoking habits during adolescence is associated with a lower risk of allergic disease [16–18].

Although the toxicity and allergenicity associated with cigarette smoke may be due to the combined action of a complex mixture of more than 4700 chemical compounds, nicotine is the main, as well as most abundant, active constituent of the pathogenic compounds in cigarette smoke [19]. Increasing evidence indicates that nicotine exerts an anti-inflammatory effect on some inflammatory diseases including ulcerative colitis and obesity [20,21], and inhibits the production of inflammatory cytokines such as interleukins (ILs) [22,23], tumor necrosis factor-α (TNF-α) [24,25] and high-mobility group box-1 (HMGB1) [26] in multiple cell types [27]. Nicotine exerts its effects through the activation of heteromeric nicotinic acetylcholine receptors (nAChRs), formed by a combination of α4 and β2 subunits, and/or homomeric nAChRs composed of α7 subunits (α7 nAChRs). These receptors exhibit high nicotine affinity and are located on non-neuronal cells [28–30]. The anti-inflammatory effects of nicotine are mediated by the activation of α7 nAChRs expressed in many different tissues and cells, including immune system cells such as macrophages and DCs, airway epithelial cells and endothelial cells [22,24,27,30]. Nicotine inhibits

Abbreviations: TSLP, thymic stromal lymphopoietin; AD, atopic dermatitis; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; ERK, extracellular signal-regulated kinase; nAChR, nicotinic acetylcholine receptor; PI3K, phosphoinositide 3-kinase; AMPK, AMP-activated protein kinase; CSE, cigarette smoke extract; TPA, 12-O-tetradecanoylphorbol-13-acetate; MLA, methyllycaconitine; α-BTX, α-bungarotoxin; MCL, mecamylamine.

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signaling by NF-κB, a pro-inflammatory transcription factor [24–26], or induces the expression of tristetraprolin (TTP) via the Janus kinase2-signal transducer and activator of transcription 3 (JAK2–STAT3) pathway, thereby increasing the decay of messenger RNA (mRNA) [31,32]. In addition, the phosphoinositide 3-kinase (PI3K) and AMP-activated protein kinase (AMPK) pathways may converge and cooperate to relay signals that intervene in the activation of nAChRs and NF-κB, and thereby interfere with the production of various cytokines [27,33,34]. However, epidermal keratinocytes are the most prevalent cell type in the skin, and the effects of nicotine on TSLP production and the mechanisms underlying its regulation have not yet been elucidated.

Therefore, we investigated the role of nicotine in epidermal TSLP expression. This is based on the hypothesis that nicotine adsorption on the skin surface through natural moisture or perspiration plays a direct role in modifying the Th1/Th2 balance in skin lesions [35]. We also examined the effects of cigarette smoke extract (CSE) and nicotine on the regulation of TSLP in the mouse keratinocyte cell line PAM212 derived from BALB/c mice. Our results showed that treatment of keratinocytes with CSE and nicotine did not induce the expression of TSLP, but did inhibit TPA-induced TSLP expression.

2. Materials and methods

2.1. Materials

12-O-Tetradecanoylphorbol-13-acetate (TPA), nicotine, and mecamylamine (MCL) were purchased from Wako Pure Chemical Ind. (Osaka, Japan). α-Bungarotoxin (α-BTX) and methyllycaconitine (MLA) were obtained from Abcam Biochemicals (Cambridge, MA, USA), and TNF-α was acquired from PeproTech, Inc. (Rocky Hill, NJ, USA). LY294002 and wortmannin were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Compound C (dorsomorphin) was acquired from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of CSE

An extemporaneous preparation of CSE was created using commercially available cigarettes (Hi-lite, Japan Tobacco Inc., nominal nicotine 1.6 mg). Briefly, 50 mL of smoke was drawn slowly through a connector into a syringe containing either 5 mL of Minimum Essential Medium alpha (MEMα) culture medium (Life Technologies, Grand Island, NY, USA) or 2 mL of deionized (DI) water and vortexed for 1 min until fully dissolved. The resulting solutions were defined as 100% and 250% CSE, respectively. CSE was then passed through a 0.22-μm Millipore filter (Millipore Corp., Bedford, MA, USA) for immediate use. The concentration of nicotine in the 100% CSE solution was estimated to be approximately 2.0 mM based upon its absorbance at 260 nm.

2.3. Cell culture

The murine keratinocyte cell line PAM212 [36] derived from BALB/c mouse skin was kindly provided by Dr. Yuspa (National Institute of Health, NCI, MD, USA), and cultured in MEMα supplemented with 10% heat-inactivated fetal bovine serum, penicillin G potassium (15 μg/mL), and streptomycin (50 μg/mL) (Meiji Seika, Tokyo, Japan). The preparation was maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere. These cells were used within five to 12 passaging cycles, and cell cultures were passaged every 3–4 days.

2.4. Treatment of cells

PAM212 cells (1×10^5 cell/mL) in MEMα medium were seeded in 6-well culture plates for the preparation of cell lysates, 12-well culture plates for RNA extraction, and 24-well culture plates for cell culture supernatants. Cell cultures at 70–80% confluency were stimulated with 30 nM TPA for an appropriate period, with or without pre-incubation with nicotine (2–200 μM) for 1 h. In some experiments, cells were pretreated 30 min before the addition of nicotine with inhibitors such as LY294002 and wortmannin for PI3K, compound C for AMPK, and α-BTX, MLA, and MCL for nAChRs. The viability of PAM212 cells was assessed using the MTT cell proliferation assay, and shown to be greater than 90% regardless of the 24 h TPA treatment applied.

2.5. Enzyme-linked immunosorbent assay (ELISA) of TSLP

At the times indicated, supernatants of the culture media were collected and stored at −30 °C. TSLP levels were assayed with the DuoSet® ELISA Development System (R&D Systems, Minneapolis, MN, USA) according to the manufacturer instructions.

2.6. Western blotting

Western blotting was performed as described previously [37]. The monoclonal antibodies (mAbs) used as the primary antibodies included: (1) phospho-IκB-α (Ser 32) Rabbit mAb (New England BioLabs Inc., Ipswich, MA, USA), (2) IκB-α (Arg 29) Rabbit mAb, (3) phospho-AMPKα (Thr172) (40H9) Rabbit mAb, AMPKα (#2532) Rabbit mAb, (4) phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Rabbit mAb, (5) p44/42 MAPK (Erk1/2) Rabbit mAb (Cell Signaling Technology, Inc., Danvers, MA, USA), and (6) NFκB p65 (C-20) Rabbit mAb (Santa Ceruz Biotechnology, Inc., Dallas, TX, USA).

2.7. Preparation of nuclear extracts

PAM212 cells stimulated with TPA (30 nM) for 45 min were used to prepare nuclear extracts with a nuclear extraction kit (Active Motif Corp, Carlsbad, CA, USA) according to the manufacturer instructions. The extracts were stored at −80 °C for subsequent analyses.

2.8. Real-time RT-PCR

Total RNA was prepared from cultured PAM212 cells at the end of the incubation period using RNAiso plus (Takara Bio Company, Shiga, Japan), and cDNA was synthesized from total RNA by reverse transcription with PrimeScript™ RT master mix (Takara) according to the manufacturer instructions. Real-time PCR was performed using SYBR® premix Ex Taq II™ (Takara) in a Thermal Cycler Dice® Real Time System TP800 (Takara). The following primers were used: GAPDH (forward: 5′-TGTGTCCGTCGTGGATCTGA-3′; reverse: 5′-TTGCTGTTGAAGTCGCAGGAG-3′), and TSLP (forward: 5′-AGCTTGTCTCCTGAAAATCGAG-3′, reverse: 5′-AGGTTTGATTCAGGCAGATGTT-3′). Relative quantification was based upon the expression levels of a target gene versus the reference GAPDH gene. Cycle threshold (Ct) values were calculated using the Second Derivative Maximum Method. Immediately after PCR, primer specificity was assessed using a melting curve analysis in the same reaction tube.

2.9. Animals

Four-week-old male BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan), raised in standard polyacrylamide cages, and housed in a specific pathogen free (SPF) animal facility