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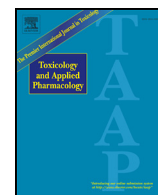

Regulation of gene expression by tobacco product preparations in cultured human dermal fibroblasts

Gloria E. Malpass [a], Subhashini Arimilli [b], G. L. Prasad [c], Allyn C. Howlett [a,*]

[a] Department of Physiology and Pharmacology, Wake Forest University Health Sciences, Winston-Salem, NC 27157, USA
[b] Department of Microbiology and Immunology, Wake Forest University Health Sciences, Winston-Salem, NC 27157, USA
[c] R&D Department, R.J. Reynolds Tobacco Company, Winston-Salem, NC 27102, USA

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ABSTRACT

Skin fibroblasts comprise the first barrier of defense against wounds, and tobacco products directly contact the oral cavity. Cultured human dermal fibroblasts were exposed to smokeless tobacco extract (STE), total particulate matter (TPM) from tobacco smoke, or nicotine at concentrations comparable to those found in these extracts for 1 h or 5 h. Differences were identified in pathway-specific genes between treatments and vehicle using qRT-PCR. At 1 h, *IL1α* was suppressed significantly by TPM and less significantly by STE. Neither *FOS* nor *JUN* was suppressed at 1 h by tobacco products. *IL8*, *TNFα*, *VCAM1*, and *NFκB1* were suppressed after 5 h with STE, whereas only *TNFα* and *NFκB1* were suppressed by TPM. At 1 h with TPM, secreted levels of IL10 and TNFα were increased. Potentially confounding effects of nicotine were exemplified by genes such as *ATF3* (5 h), which was increased by nicotine but suppressed by other components of STE. Within 2 h, TPM stimulated nitric oxide production, and both STE and TPM increased reactive oxygen species. The biological significance of these findings and utilization of the gene expression changes reported herein regarding effects of the tobacco product preparations on dermal fibroblasts will require additional research.

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Introduction

Cigarette smoke exerts both systemic and direct effects at local areas of exposure including the oral cavity (Sopori, 2002; van der Vaart et al., 2004). Exposure to cigarette smoke or its constituent phases alters cytokine expression in various cell types, e.g. human aortic endothelial cells, THP-1 monocyte macrophages (Nordskog et al., 2005), and normal human bronchial epithelial cells (NHBEs) (Fields et al., 2005). The particulate phase of cigarette smoke, total particulate matter (TPM), also commonly known as cigarette smoke condensate (CSC), uniquely influences the expression of cytokines depending on cell type (Nordskog et al., 2005).

Acute exposure to cigarette smoke or its constituent phases can cause oxidative stress, inflammation, and cytotoxicity. Exposure to TPM induces: the cytochrome P450 family of genes in normal epidermal keratinocytes, oral dysplasia cells, and primary oral carcinoma cells (Nagaraj et al., 2006); the hemoxygenase gene in human alveolar epithelial cells (Fukano et al., 2006); cytokines including interleukin-8 (*IL8*) in primary human peripheral blood mononuclear cells, human lymphoblasts (HL60) (Arimilli et al., 2012), and NHBEs (Parsanejad et al., 2008); and caspases in THP-1 monocytes (Arimilli et al., 2012), oral squamous cell carcinoma cells, and normal human gingival epithelial cells (Gao et al., 2013). Gene expression/transcriptomic changes of cigarette smoking have been proposed as biomarkers of lung cancer, chronic obstructive pulmonary disease, and other smoking-related diseases (Gower et al., 2011).

Smokeless tobacco (ST) products comprise a large and diverse category of tobacco products consumed worldwide. The European and US studies indicate that ST consumption, relative to smoking, is associated with significantly reduced risk of serious diseases including lung cancer, oral cancer, and chronic obstructive pulmonary disease (Lee and Hamling, 2009). However, information on cellular responses following exposure to ST preparations is relatively limited, and the cellular effects appear to depend on how the ST extracts are prepared. Recent reports (Arimilli et al., 2012; Gao et al., 2013) show that combustible tobacco product preparations are far more cytotoxic than ST preparations. Information on the comparative effects of ST and combustible tobacco (e.g., TPM) preparations on gene expression is also limited and would be valuable in understanding biological and pathophysiological effects of different tobacco product categories.

TPM upregulates gene and protein expression of pro-inflammatory cytokines including *IL1α*, *IL1β*, *IL6*, and *IL8* in human fibroblast-like synoviocyte cells (Shizu et al., 2008) and initiates an inflammatory response in vocal fold fibroblasts (Branski et al., 2011). This study

* Corresponding author at: Department of Physiology and Pharmacology, Wake Forest University Health Sciences, Medical Center Boulevard, Winston-Salem, NC 27157, USA. Fax: +1 336 713 1545.
E-mail addresses: gloria.malpass@gmail.com (G.E. Malpass), sarimill@wakehealth.edu (S. Arimilli), prasadg@rjrt.com (G.L. Prasad), ahowlett@wakehealth.edu (A.C. Howlett).

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examined immediate early gene expression changes, secreted cytokine levels, and oxidative stress in actively growing normal adult human dermal fibroblasts (HDFa) in culture as a model for comparing the relative effects of smokeless tobacco extract (STE), TPM from reference cigarettes, and nicotine at non-cytotoxic levels.

Methods

Materials

Reagents and cell culture materials were purchased from the following sources: normal adult human dermal fibroblasts, fibroblast basal media (FBM), fibroblast supplemental growth factors (hydrocortisone hemisuccinate, HLL supplements [human serum albumin, linoleic acid, and lecithin], rh FGFβ, rh EGF/TGF, β-1 supplement, rh insulin, and ascorbic acid), and 0.1% gelatin solution, American Type Culture Collection (ATCC; Manassas, VA); fetal bovine serum, Atlanta Biologicals, Inc. (Lawrenceville, GA); GlutaMAX I and TRIzol, Life Technologies (Grand Island, NY); Dulbecco's Phosphate Buffered Saline (DPBS), Lonza (Walkersville, MD); penicillin (100 units$\cdot$mL^{-1}) and streptomycin (100 μg$\cdot$mL^{-1}) solution (Pen–Strep), phenol red, and dimethyl sulfoxide (DMSO), Sigma-Aldrich (St. Louis, MO); β-mercaptoethanol, Calbiochem, (La Jolla, CA); HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Thermo Fisher Scientific (Waltham, MA); and DAF-FM diacetate, Life Technologies (Grand Island, NY). A 5 mM stock solution of DAF-FM diacetate was prepared by diluting DAF-FM diacetate with GMP Biotechnology Performance Certified anhydrous DMSO.

TPM, STE, and CAS were prepared by Labstat International, Kitchener, Ontario, Canada (Arimilli et al., 2012; Gao et al., 2013). CAS was prepared using a protocol based on the work of Chou and Hee (1994). Components in CAS were: mucin (1.35 g/l), potassium chloride (475 mg/l), sodium chloride (700 mg/l), calcium chloride dehydrate (185 mg/l), di-potassium hydrogen phosphate (420 mg/l), magnesium chloride hexahydrate (105 mg/l), urea (45 mg/l), D-(+)-glucose (100 mg/l), α-amylase (100,000 U/l), lysozyme (750 U/l), and acid phosphatase (4 U/l). STE was prepared by extracting 2.5 g of 2S3 smokeless tobacco reference product (North Carolina State University Tobacco Services Analytical Laboratory) in 25 ml CAS for 2 h followed by filtration to produce a 10% STE stock solution. CAS served as the solvent control for all experiments using STE. TPM was prepared by smoking 3R4F reference cigarettes (University of Kentucky), collecting the particulate phase on glass fiber, and dissolving the particulate in DMSO. For experiments using TPM, DMSO served as the solvent control. Chemical analyses for nicotine and tobacco specific nitrosamine contents were performed by Labstat.

Kits for assays were purchased from the following sources: Qiashredder columns, Qiagen RNeasy mini kits, RT^2 Profiler PCR Arrays for Human Transcription Factors and for Human Signal Transduction PathwayFinder, RT^2 First Strand Kits, RT^2 SYBR Green qPCR Mastermix, and RNase-Free DNase Sets, Qiagen Inc. (Valencia, CA); high capacity cDNA reverse transcription kits and TaqMan Expression Assays, Applied Biosystems (Foster City, CA); FastStart Universal Probe Master (Rox), Roche (Indianapolis, IN); Cytometric Bead Array (CBA) human inflammatory cytokine kits, BD Biosciences (San Jose, CA); and total reactive oxygen and nitrogen species (RO/NS) and superoxide detection kits, Enzo Life Sciences Inc. (Farmingdale, NY).

Cell culture and treatments

HDFa were maintained in serum-complete media comprised of FBM supplemented with 10% FBS, GlutaMAX I (2 mM), Pen–Strep (1:100 dilution), and phenol red (2 μM) in a humidified 5% CO_2 incubator at 37 °C. HDFa were plated at a density of 4×10^5 cells in 60 mm tissue culture dishes in serum-complete media, such that cells were 50% to 80% confluent and actively proliferating at the time of each experiment. After 20 to 24 h, serum-complete media were removed, cells were rinsed twice with DPBS, and media were changed to serum-free defined media comprised of (1) FBM, GlutaMAX I, Pen–Strep, phenol red, and fibroblast supplemental growth factors (hydrocortisone hemisuccinate [1 μg/ml], human serum albumin [500 μg/ml], linoleic acid [0.6 μM], lecithin [0.6 μg/ml], rh FGFβ [5 ng/ml], rh EGF/TGF [5 ng/ml], β-1 supplement [30 pg/ml], rh-insulin [5 μg/ml], and ascorbic acid [50 μg/ml]), or (2) FBM, GlutaMAX I, Pen–Strep, and phenol red. Appropriate test article or vehicle was added, and HDFa cells were exposed to these products at 37 °C and 5% CO_2 for 1 h or 5 h. At the designated time following treatment, cells were collected for RNA isolation, or media were collected for the determination of cytokine release.

For treatments, 1% STE was prepared by diluting 10% STE in CAS to yield a solution with 141.6 μg/ml nicotine content, a concentration within the range of salivary levels (70 to 1560 μg/ml) in ST users (Petro, 2003). Nicotine hydrogen tartrate salt (NIC) was dissolved in CAS to yield a solution with a nicotine concentration of 141.6 μg/ml (NIC/CAS). Equivalent volumes of CAS, STE/CAS, and NIC/CAS were administered to cells. TPM was administered at a nicotine concentration of 4 μg/ml, a concentration found to be non-cytotoxic for time periods ≤24 h (Gao et al., 2013) and within the range of salivary levels (0.9 to 4.6 μg/ml) in smokers (Robson et al., 2010). NIC was dissolved in DMSO to yield a solution with a nicotine concentration of 4 μg/ml (NIC/DMSO). Equivalent volumes of DMSO, TPM, and NIC/DMSO were administered to cells.

RNA isolation and cDNA preparation

The cell monolayer was rinsed twice with DPBS and dislodged with TRIzol containing 1% β-mercaptoethanol. Cells were transferred to Qiashredder columns and disrupted by sedimentation at 14,000 rpm for 2 min. Supernatant was collected and RNA was isolated using the Qiagen RNeasy mini kit. Purity and quantity of RNA were measured on the NanoDrop 2000 (Thermo Fisher Scientific Inc., Wilmington, DE). For RT^2 Profiler PCR Arrays, RNA isolation included an on-column DNase digestion using an RNase-Free DNase Set (Qiagen) and total RNA was converted into first strand cDNA while genomic DNA was also removed from the RNA using an RT^2 First Strand Kit comprised of genomic DNA elimination buffer, RT buffer, primer and external control mix, RT enzyme mix, and RNase-free H_2O. For follow-up experiments, total RNA was converted to single-stranded cDNA using a high capacity cDNA reverse transcription kit comprised of RT buffer, RT random primers, dNTP mix, reverse transcriptase, and RNase-free H_2O.

Gene expression arrays

For array detection of gene expression differences between groups, qRT-PCR analyses were performed using RT^2 Profiler Arrays and RT^2 SYBR Green qPCR Mastermix. Two sets of arrays were performed: (1) Human Signal Transduction PathwayFinder; and (2) Human Transcription Factors. All data were normalized to the average of three housekeeping genes: 60s ribosomal protein L13a (*RPL13A*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and beta-actin (*ACTB*). Fold changes are reported relative to vehicle control. All data were analyzed using the RT^2 Profiler PCR Array Data Analysis Web Portal (Qiagen) for converting cycle time to fold change.

For follow-up experiments, qRT-PCR analyses were performed using TaqMan Expression Assays and FastStart Universal Probe Master (Rox). Target genes included: *FOS*, *IL1α*, *JUN*, *IL8*, *TNFα*, *VCAM1*, *NFκB1*, and *ATF3*. Three to five independent experiments with two technical replicates for each were performed. All data were normalized to the average of three housekeeping genes: *RPL13A*, *GAPDH*, and *ACTB*. Fold changes are reported relative to basal control (at time $t = 0$ h). Comparisons between treated and vehicle values were analyzed using REST 2009 (Relative Expression Software Tool V2.0.13, Qiagen, Inc., Valencia, CA). This software tool compares a sample group and control group, and

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