ORIGINAL RESEARCH LETTER

Effect of long-acting β-agonists/glucocorticoids on human airway epithelial cell cytokine, transcriptomic and oxidative stress responses to cannabis smoke

To the Editor:

Cannabis is one of the most widely consumed drugs in the world, with an estimated 180 million annual users globally [1]. Recent trends of legalisation of medicinal and/or recreational cannabis have led to increased access to cannabis products that will likely lead to increased use over the coming years. Inhalation of combusted cannabis is by far the most prevalent form of cannabis use, reported by 89% of users in the 2018 Canadian Cannabis Survey [2]. Chemically, cannabis smoke includes pharmacologically active components such as tetrahydrocannabinol (THC), cannabidiol (CBD) and polycyclic aromatic hydrocarbons [3]. Many studies have investigated the psychoactive and immunomodulatory properties of THC and CBD respectively, and examined the potential use of cannabis as an intervention for chronic pain, immune disorders and neurological disorders [4, 5]. Despite this body of knowledge and recent rise of systematic reviews on these topics, the effects of inhaled cannabis smoke on the respiratory mucosal immune responses are less clear [6].

Like tobacco smoke, repeated exposure to cannabis smoke has been associated with cough and shortness of breath [7]. However, few studies have examined the effects of cannabis smoke on the respiratory mucosa, the primary physical and immunological barrier to the environment [8]. Tobacco smoke induces oxidative stress and cytokine production while disrupting epithelial barrier function, impacting chronic lung disease pathology and exacerbations [9]. To manage chronic lung diseases, an individual may be prescribed a broad, anti-inflammatory long-acting β-agonist (LABA)/glucocorticoid (GC) combination treatment [10], a therapy that may be compromised by tobacco smoke exposure [11, 12]. Given the similarities between tobacco and cannabis smoke [8], we examined whether cannabis smoke exposure similarly attenuates LABA/GC transcriptomic responses and inflammatory mediator release in human airway epithelial cells.

Cannabis smoke extract (CSE)- and tobacco smoke extract (TSE)-conditioned media were prepared according to previously published methods [12]. TSE was generated from Kentucky Research Grade Cigarettes with an intact filter (lot 3R4F), and CSE from cannabis rolled with cardboard filters and sourced from Jonathan Page (University of British Columbia, Vancouver, Canada) (13% (w/w) tetrahydrocannabinolic acid strain with 0.18% THC, 0.35% tetrahydrocannabivarinic acid and 0.18% cannabigerolic acid; ∼0.7 g dried cannabis). Extracts were prepared by bubbling cannabis or tobacco smoke through 4 mL HEPES-buffered Eagle's minimal essential medium, filtering (0.22 µm) and diluting with fresh medium (10% dilution, optical density at 260 nm 0.04045). Calu-3 lung epithelial cells on Transwells (Corning Inc., Corning, NY, USA) were apically exposed to 10% of either CSE or TSE with and

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LABA/GC intervention in airway epithelial cells exposed to cannabis smoke reduces levels of pro-inflammatory (CXCL8) and antiviral (CXCL10) mediators, while transcriptomic signatures of neutrophil-mediated immunity and oxidative stress remain elevated http://bit.ly/2qiSQhH

Cite this article as: Huff RD, Aguiar JA, Tse W, *et al.* Effect of long-acting β-agonists/glucocorticoids on human airway epithelial cell cytokine, transcriptomic and oxidative stress responses to cannabis smoke. *ERJ Open Res* 2020; 6: 00265-2019 [https://doi.org/10.1183/23120541.00265-2019].

without 10 nM formoterol and 100 nM budesonide (Form/Bud) for 24 h. Total RNA was extracted and sent to The Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada) for RNA sequencing. Apical cell supernatants were assessed using a multiplex cytokine/chemokine protein array by Eve Technologies (Calgary, Canada). Oxidative stress was assessed using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and CellROX Green (Thermo Fisher Scientific, Waltham, MA, USA), counterstained using Hoechst 3342 and quantified using a plate reader. All experiments had four biological replicates. Significant differences in cytokines and oxidative stress were identified through permutation ANOVA followed by a Tukey Honest Significant Difference *post hoc* test using the "lmPerm" package in R (version 3.4.3; The R Foundation, Vienna, Austria). Significant differences between transcriptional expression profiles were identified through analysis of similarities using the "vegan" package in R.

Exposure to 10% CSE or TSE and/or Form/Bud for 24 h did not significantly change cell viability as assessed by lactate dehydrogenase release assays (not shown). Like TSE, CSE significantly elevated proinflammatory cytokine interleukin-8/CXCL8 and epithelial cell repair mediator transforming growth factor-α (figure 1a and b). Intervention with Form/Bud prevented CSE and TSE increases in IL-8/CXCL8 release and, to a lesser extent, TGF-α. We also observed a significant decrease in interferon-γ-induced protein 10/CXCL10 (figure 1c) in response to CSE and TSE exposure, a decrease that was augmented in response to Form/Bud intervention.

We next interrogated RNA sequencing data to address the efficacy of LABA/GC intervention on attenuating CSE- and TSE-induced transcriptomic responses observed in our previous study [8]. First, we observed that the most upregulated gene exclusive to Form/Bud treatment was *HSD11B2* (corticosteroid 11-β-dehydrogenase) (vehicle+Form/Bud FC 34.80, CSE+Form/Bud FC 33.99 and TSE+Form/Bud FC 27.32), indicating that our intervention was efficacious on modulating glucocorticoid signalling (data not shown). Second, to identify expression patterns unique to each smoke exposure+LABA/GC intervention, we directly compared these datasets to identify differentially expressed genes and visualised their $\log_2$ fold changes relative to untreated+Form/Bud (figure 1d). Form/Bud intervention in smoke-exposed cells induced a highly correlated transcriptomic response ($r=0.771$, $p<1.0\times10^{-15}$), with 801 differentially expressed genes in CSE+Form/Bud *versus* untreated+Form/Bud and 1105 differentially expressed genes in TSE+Form/Bud *versus* untreated+Form/Bud. Third, *NEU1* was the most significantly increased gene in both CSE+Form/Bud and TSE+Form/Bud compared to vehicle+Form/Bud exposed cells, and aryl hydrocarbon receptor (AHR) genes *CYP1A1* and *CYP1B1* remained upregulated in both CSE+Form/Bud- and TSE+Form/Bud-exposed cells.

To further interrogate genes significantly differentially expressed in both CSE+Form/Bud- and TSE+Form/Bud-exposed cells, we performed a functional enrichment analysis (figure 1e). Interestingly, regulation of apoptotic process (rank 1, 45 out of 816 genes, gene ontology (GO):0042981), neutrophil-mediated immunity (rank 3, 30 out of 108 genes, GO:0002446), and cellular response to oxidative stress (rank 6, 13 out of 488 genes, GO:0034599) were included in the top 10 GO terms ranked by adjusted p-value. To functionally characterise the "cellular response to oxidative stress" term, we performed additional *in vitro* experiments and analysis of oxidative stress-related gene expression data [13, 14] (figure 1f). Interestingly, several curated genes (*e.g. GCLC* and *GCLM)* were increased beyond CSE or TSE exposure with Form/Bud intervention. Furthermore, when we examined overall oxidative stress using CellROX (figure 1g–m) and H_2DCFDA (figure 1n) *in vitro* imaging assays, we observed reactive oxygen species (ROS) generation in both CSE+Form/Bud- or TSE+Form/Bud-exposed cells.

Tobacco smoke has long been associated with increased symptoms and severity of chronic lung diseases that include asthma, pulmonary fibrosis and COPD, and attenuated GC responses in human lung epithelial cells [12]. Chronic bronchitis and airway remodelling/scarring due to persistent inflammation and oxidative stress from recurring exposure to tobacco smoke are known to be important pathological mechanisms in the progression of COPD and asthma [11, 15]. We demonstrate here for the first time that a Form/Bud intervention of human lung cells exposed to cannabis smoke suppressed increased inflammation (IL-8/CXCL8) and epithelial repair mediators (TGF-α), while expression of oxidative stress genes remained elevated. Although Form/Bud intervention reduced the expression of IL-8/CXCL8, CSE and TSE exposure may still be modulating epithelial immune responses, as seen by the continued enrichment of neutrophil-related GO terms. Furthermore, the persistence of increased generation of ROS and expression of the Nrf2 oxidative stress response genes in the presence of LABA/GC medications used in chronic respiratory disease management may further impact long-term disease development and management.

Our study has several limitations in the experimental design. Our model is a single exposure to smoke-conditioned media and may not accurately represent chronic smoke exposure conditions, which

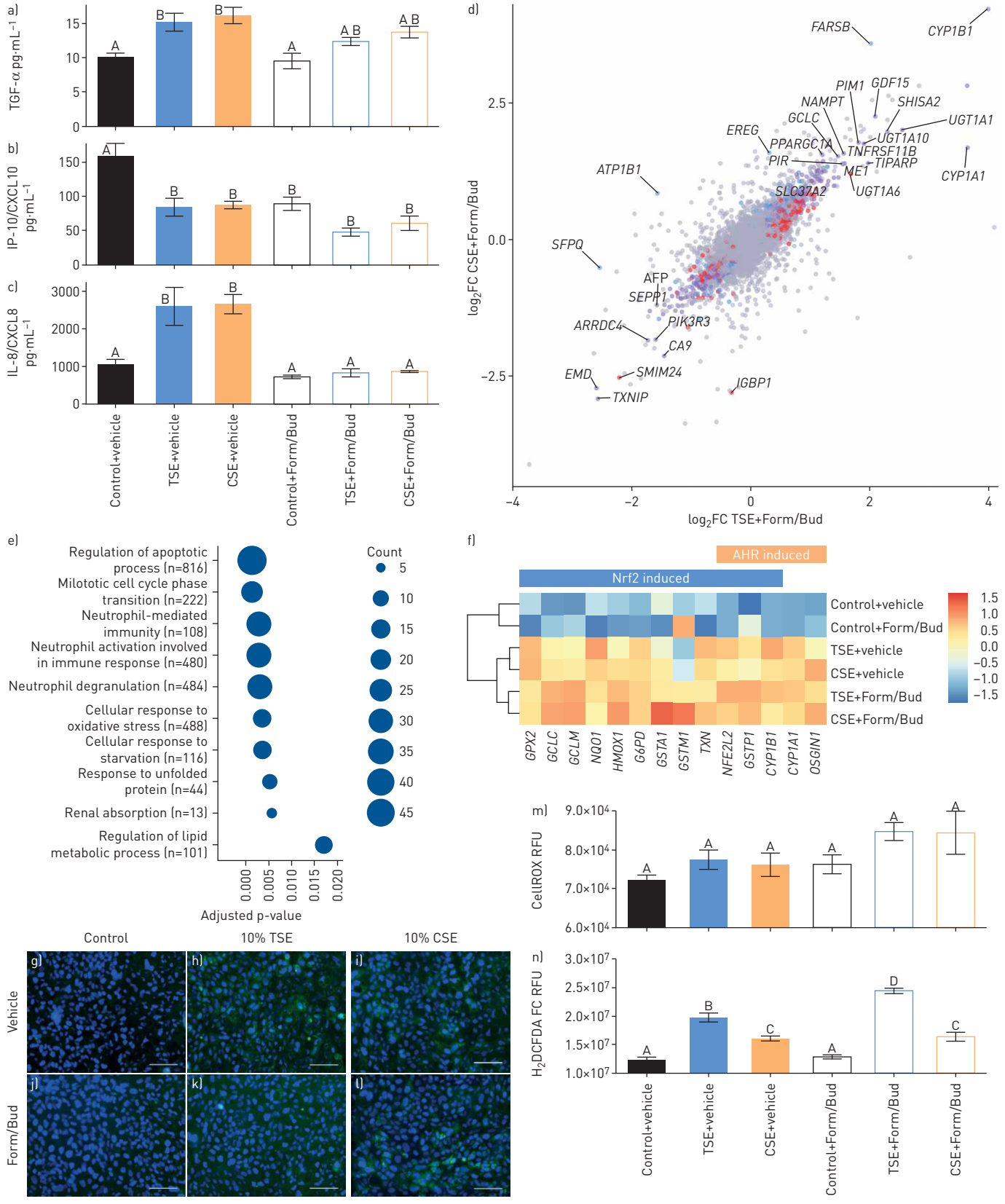

FIGURE 1 Responses of Calu-3 cells to 10 nM formoterol and 100 nM budesonide (Form/Bud) were examined in the context of cannabis smoke extract (CSE)-conditioned media. Calu-3 cells were exposed to either control, 10% CSE (orange) or 10% tobacco smoke extract (TSE) (blue) with either vehicle or Form/Bud for 24 h. To assess the impact of Form/Bud on inflammatory mediators, cytokine release of a) transforming growth factor (TGF)-α, b) interferon-γ-induced protein (IP)-10/CXCL10 and c) interleukin (IL)-8/CXCL8 was measured. d) The effect of Form/Bud intervention and CSE or TSE exposure on airway epithelial gene expression was assessed by RNA sequencing. The $\log_2$ fold change (FC) of

CSE+Form/Bud *versus* vehicle+Form/Bud was plotted against the log_2FC of TSE+Form/Bud *versus* vehicle+Form/Bud to reveal significant TSE- and CSE-specific genes under Form/Bud intervention conditions. r=0.771; $p<1\times10^{-15}$. Genes that were significantly differentially expressed (q<0.05) in CSE+Form/Bud and TSE+Form/Bud *versus* vehicle+Form/Bud are highlighted in purple, where as CSE- and TSE-specific genes are highlighted in blue and red respectively. To examine the similarity of CSE+Form/Bud and TSE+Form/Bud transcriptomic profiles, correlation of differential gene expression was determined by Pearson's correlation. e) Functional enrichment analysis of genes shown to be significantly differentially expressed (q<0.05) in CSE+Form/Bud and TSE+Form/Bud *versus* vehicle+Form/Bud. Top 10 gene ontology biological processes were ranked by decreasing adjusted p-value, with number of significantly differentially expressed genes (count) contributing to the total number of genes associated with the given pathway (n) denoted by the size of circle. f) Expression of genes involved in NRF2 and AHR responses. g–l) Oxidative stress was assessed using CellROX and m) the fluorescent intensity was quantified n) with complementary quantification using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). All experiments were performed using four biological replicates from serial passages. Different letters indicate significantly different group means (Tukey Honest Significant Difference, p<0.05). There are no significant differences between means sharing a common letter. Error bars represent SEM. Scale bars=100 µm. RFU: relative fluorescence unit.

would require repeated exposures over time *in vitro*. Despite this limitation, we emphasise that our previous publication using this model demonstrated strong significant correlations of gene expression with *in situ* bronchial brushes from human smokers and primary air–liquid interface cultures exposed to tobacco smoke [8]. Additional experiments using primary human airway epithelial cells grown under air–liquid interface culture conditions and exposed to whole-smoke extract should be explored to complement the present data. We recognise that there are substantial differences in cannabis strains available on the medicinal and recreational market, and therefore chose a representative hybrid strain available on the medicinal cannabis market in Canada. Despite these limitations, the robust changes we observed in our data suggest that cannabis smoke exposure still poses a significant health risk, and warrants ongoing study to build a body of evidence to support public policy, government regulations and individual user practices.

Ryan D. Huff[1,5], Jennifer A. Aguiar[2,5], Wayne Tse[1], Martin R. Stämpfli[3,4], Brendan J. McConkey[2,3], Andrew C. Doxey[2,3,6] and Jeremy A. Hirota[1,2,3,4,6]

[1]Division of Respiratory Medicine, Dept of Medicine, University of British Columbia, Vancouver, BC, Canada. [2]Dept of Biology, University of Waterloo, Waterloo, ON, Canada. [3]Firestone Institute for Respiratory Health – Division of Respirology, Dept of Medicine, McMaster University, Hamilton, ON, Canada. [4]McMaster Immunology Research Centre, Dept of Pathology and Molecular Medicine, McMaster University, Hamilton, ON, Canada. [5]Joint first authors. [6]Joint senior authors.

Correspondence: Jeremy A. Hirota, Firestone Institute for Respiratory Health – Division of Respirology, Dept of Medicine, McMaster University, Hamilton, ON, L8N 4A6, Canada. E-mail: hirotaja@mcmaster.ca

Received: 25 Sept 2019 | Accepted after revision: 18 Nov 2019

Conflict of interest: None declared.

Support statement: This study was supported by the McMaster University Centre for Medicinal Cannabis Research. Funding information for this article has been deposited with the Crossref Funder Registry.

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