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Diacetyl and 2,3-pentanedione exposure of human cultured airway epithelial cells: Ion transport effects and metabolism of butter flavoring agents

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ABSTRACT

Inhalation of butter flavoring by workers in the microwave popcorn industry may result in "popcorn workers' lung." In previous in vivo studies rats exposed for 6 h to vapor from the flavoring agents, diacetyl and 2,3-pentanedione, acquired flavoring concentration-dependent damage of the upper airway epithelium and airway hyporeactivity to inhaled methacholine. Because ion transport is essential for lung fluid balance, we hypothesized that alterations in ion transport may be an early manifestation of butter flavoring-induced toxicity. We developed a system to expose cultured human bronchial/tracheal epithelial cells (NHBEs) to flavoring vapors. NHBEs were exposed for 6 h to diacetyl or 2,3-pentanedione vapors (25 or ≥60 ppm) and the effects on short circuit current and transepithelial resistance (R_t) were measured. Immediately after exposure to 25 ppm both flavorings reduced Na^+ transport, without affecting Cl^- transport or Na^+,K^+-pump activity. R_t was unaffected. Na^+ transport recovered 18 h after exposure. Concentrations (100–360 ppm) of diacetyl and 2,3-pentanedione reported earlier to give rise in vivo to epithelial damage, and 60 ppm, caused death of NHBEs 0 h post-exposure. Analysis of the basolateral medium indicated that NHBEs metabolize diacetyl and 2,3-pentanedione to acetoin and 2-hydroxy-3-pentanone, respectively. The results indicate that ion transport is inhibited transiently in airway epithelial cells by lower concentrations of the flavorings than those that result in morphological changes of the cells in vivo or in vitro.

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1. Introduction

Microwave popcorn manufacturing employees who inhale butter flavoring vapor can develop "popcorn worker's lung," an obstructive pulmonary disease which resembles clinical bronchiolitis obliterans (Kreiss et al., 2002) in which the airway epithelium is the apparent initial target of injury (Hubbs et al., 2002, 2008, 2012; Morris and Hubbs, 2009; Morgan et al., 2008, 2012; Palmer et al., 2011). In animal models of popcorn worker's lung the airway epithelium of the upper airways is a site, in morphological terms, of cytotoxicity. Inhalation of volatile α-diketone components in popcorn flavoring mixtures, such as diacetyl and 2,3-pentanedione, leads to appreciable epithelial damage in animal inhalation models and this effect is thought to be a primary contributing factor to the development of popcorn worker's lung (Akpinar-Elci et al., 2004; Day et al., 2011; Fedan et al., 2006; Hubbs et al., 2002; Kreiss et al., 2002; Lockey et al., 2009; Morgan et al., 2008, 2012; Morris and Hubbs, 2009; Morris, 2012; Palmer et al., 2011; van Rooy et al., 2007, 2009).

Regulation of the airway surface liquid (ASL) is one of the most important functions of the epithelium (Hollenhorst et al., 2011). In humans, epithelial cells throughout the respiratory tract are involved in Na^+ absorption and Cl^- secretion as a means of regulating the height of ASL and mucociliary clearance (Hamann et al., 2010; Hollenhorst et al., 2011). Na^+ absorption occurs on the apical surface through amiloride-sensitive Na^+ channels and apical Cl^- channels subserve apical Cl^- secretion. At the basolateral surface of the cell, Na^+ is pumped outwardly by the Na^+,K^+-pump (Matthay et al., 2002). Ion transport disruption may result in respiratory infection and diseases including cystic fibrosis (CF) and pulmonary edema (Harris, 1996; Hollenhorst et al., 2011), as well as airway obstruction (Danahay et al., 2002; Eisenhut, 2006; Houtmeyers et al., 1999). Reduced transepithelial Na^+ transport in the airways has been demonstrated to result in pulmonary edema (Egli et al., 2004). Previous findings using the guinea-pig

Abbreviations: ASL, airway surface liquid; CFTR, cystic fibrosis transmembrane conductance regulator; ECC, exposure chamber control; NHBEs, normal human bronchial/tracheal epithelial cells; ENaC, epithelial sodium channel; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; I_{sc}, short circuit current; R_t, transepithelial resistance; TEM, transmission electron microscopy; SEM, scanning electron microscopy; DCXR, dicarbonyl/L-xylulose reductase; MKHS, modified Krebs–Henseleit solution.

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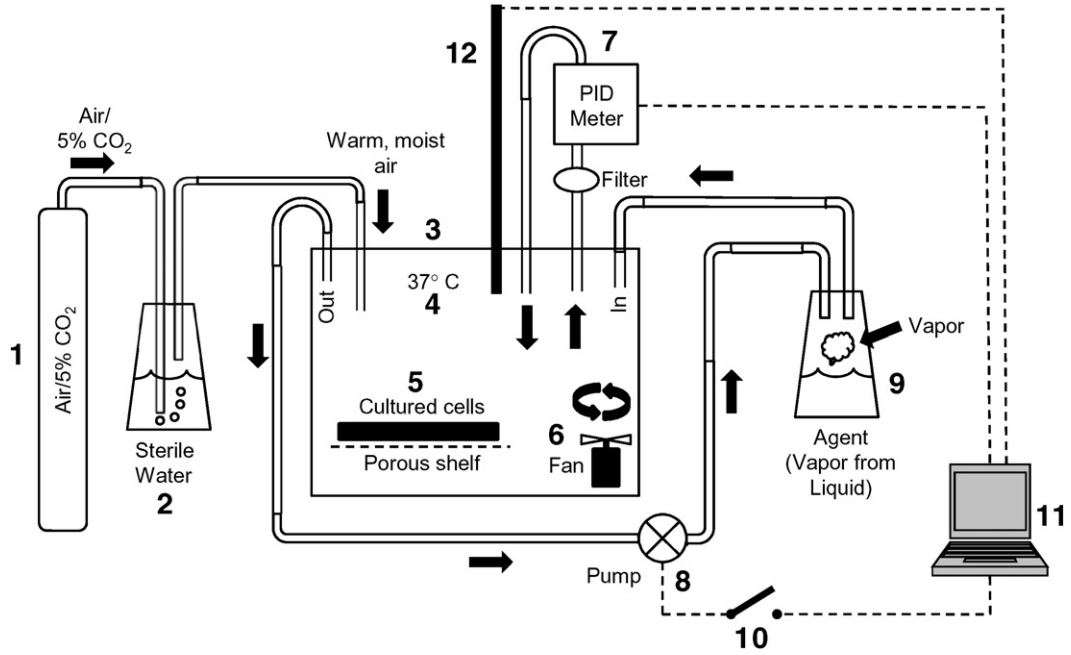

Fig. 1. Schematic of the apparatus designed and used for exposing ALI NHBEs to vapors of diacetyl delivered to the apical surface. 1: Gas (5% CO_2 in air) for providing oxygenation of cells and maintaining pH of the culture medium. 2: Passage of gas through sterile, distilled water (37 °C) to humidify the gas to 85% relative humidity. 3: The exposure chamber. 4: Chamber temperature was maintained at 37 °C. The exposure chamber mimicked conditions in the incubator in which the cells were grown and differentiated. 5: Cultured cell plate containing NHBEs rested on a porous stainless steel shelf. 6: Fan to ensure mixing of agent in the chamber. 7: Photoionization detector (PID) for measuring vapor level. The PID withdrew air from the chamber and returned it while measuring vapor level in the chamber. The output of the PID was sent to the proprietary computer software (11) for integration. 8: A pump was activated/deactivated by the computer in response to input from the PID. The pump delivered vapors to the chamber. 9: Vapor source from which vapor is pumped to the exposure chamber upon activation of the pump. The vapor was generated from diacetyl or 2,3-pentanedione liquid placed in the bottom of the vessel. 10: The computer software activated/deactivated a switch to engage or disengage the pump. 11: Proprietary software was used to monitor vapor levels in the chamber and deliver vapor as needed to maintain the level within 5% of the desired level. 12: The temperature-humidity probe was monitored by computer software.

isolated, perfused system revealed that diacetyl administered to the lumen via the perfusing physiological salt solution led to transepithelial depolarization and altered tight junction integrity (Fedan et al., 2006). In view of the importance of ion transport to normal lung function, we reasoned that understanding the effect(s) of flavorings on ion transport is needed, as they might be involved in epithelial responses to flavorings. At present the effects flavorings delivered directly to NHBE cells in vitro are unknown. In order to accomplish this characterization, a high throughput device was developed for inhalation exposure of NHBEs to diacetyl and 2,3-pentanedione.

It was heretofore not known which ion transporters might be affected by the butter flavoring agents, and we wanted to clarify a possible relationship between altered ion transport and epithelial cellular injury, and to understand the direct effects of butter flavoring agents on airway epithelial cells that are not adherent to the airway wall and not under the influence of other cell types and their mediators. This includes the question as to whether NHBEs were capable of metabolizing diacetyl and 2,3-pentanedione.

There were two primary goals of this investigation. The first was to evaluate the effects of diacetyl and 2,3-pentanedione on epithelial cell ion transport following exposure to 25 ppm of flavoring vapors in vitro, in comparison to concentrations that evoke morphological damage, as well as concentrations used in our previous vivo studies (60–360 ppm; Zaccone et al., 2013). The second was to investigate the ability of intact NHBE cells to metabolize diacetyl and 2,3-pentanedione following exposure to vapors in a novel in vitro exposure system. Diacetyl and 2,3-pentanedione are metabolized by dicarbonyl/l-xylulose reductase (DCXR; Nakagawa et al., 2002; Ebert et al., 2014). Gloede et al. (2011) and Cichocki et al. (2014) measured diacetyl levels and metabolites of diacetyl in upper and lower lung tissues as a component of a computational fluid dynamics-physiologically based pharmacokinetic (CFD-PBPK) model of diacetyl distribution and handling in the airways.

2. Materials and methods

2.1. Cell culture

Primary cultured NHBEs (Lonza; Walkersville, MA) were seeded in plastic T-75 flasks and were grown in B-ALI growth medium (Lonza) until cells were 80% confluent. The confluent monolayer was trypsinized and seeded onto rat tail collagen (BD Biosciences; San Jose, CA)-coated polyester (0.4 μm pores) transwell inserts (Corning; Corning, NY) at a density of 50,000 cells per insert. Cells were maintained at 37 °C in an air/5% CO_2 mixture in an incubator. Cells were submerged for three days in B-ALI growth medium (100 μl apical; 500 μl basal chamber) before 500 μl of B-ALI differentiation medium was added to the basal chamber and the apical chamber was emptied to initiate the air-liquid interface (ALI) culture conditions. Medium was changed every 48 h. Transepithelial resistance (R_t) was measured with EVOM^2 epithelial volt-ohm meter STX^2 electrodes (World Precision Instruments; Sarasota, FL) to assess growth to confluence from the increase in the R_t. Cells were used after R_t reached a value of at least 700 $\Omega \cdot cm^2$, which occurred after 7 days.

2.2. Imaging of cultured NHBEs

Differentiated, pseudostratified epithelium morphology was confirmed through a series of imaging and staining techniques. For hematoxylin and eosin (H&E) staining the transwell inserts were fixed in 10% buffered formalin, rinsed in Hank's balanced salt solution (37 °C), dehydrated in a graded series of ethanol, cleared in xylene, and infiltrated and embedded in paraffin. Sections (5 μm) were placed on microscope slides and stained with H&E. The samples were imaged on an Olympus IX70 photomicroscope (Shinjuku, Tokyo).

Mucus production by NHBEs was confirmed using alcian blue staining. Transwell inserts were stained apically with a 1% alcian blue