Toxicology Letters 325 (2020) 25–33

Contents lists available at ScienceDirect

Toxicology Letters

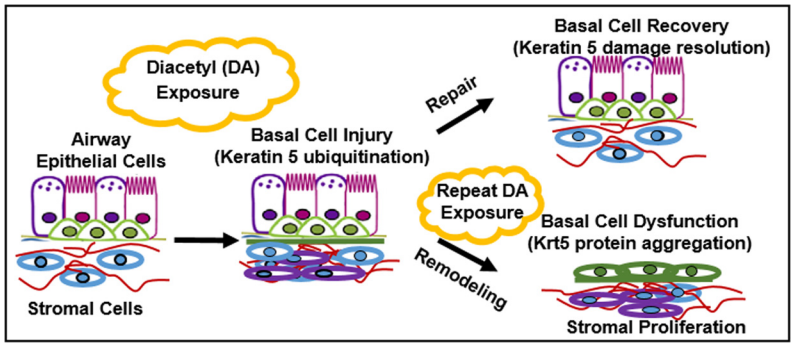
journal homepage: www.elsevier.com/locate/toxlet

Airway basal cell injury after acute diacetyl (2,3-butanedione) vapor exposure

Matthew D. McGraw[a,b,*], So-Young Kim[a], Christina Reed[b,c], Eric Hernady[b], Irfan Rahman[b], Thomas J. Mariani[a,c,d], Jacob N. Finkelstein[b,c]

[a] Department of Pediatrics, Division of Pulmonology, Rochester, NY, United States
[b] Department of Environmental Medicine, Rochester, NY, United States
[c] Department of Pediatrics, Division of Neonatology, Rochester, NY, United States
[d] Department of Pediatrics, Program in Pediatric Molecular and Personalized Medicine, University of Rochester Medical Center, Rochester, NY, United States

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Diacetyl
Airway epithelium
Keratin 5
Ubiquitin proteasome
Epithelial repair
Flavoring-Induced lung disease

ABSTRACT

Rationale: Diacetyl (DA; 2,3-butanedione) is a chemical found commonly in foods and e-cigarettes. When inhaled, DA causes epithelial injury, though the mechanism of repair remain poorly understood. The objective of this study was to evaluate airway basal cell repair after DA vapor exposure.
Methods: Primary human bronchial epithelial cells were exposed to DA or PBS for 1 h. Lactate dehydrogenase, cleaved caspase 3/7 and trans-epithelial electrical resistance were measured prior to and following exposure. Exposed cultures were analyzed for the airway basal cell markers keratin 5 and p63 as well as ubiquitin and proteasome activity. Cultures were also treated with a proteasome inhibitor (MG132).
Results: DA vapor exposure caused a transient decrease in trans-epithelial electrical resistance in all DA-exposed cultures. Supernatant lactate dehydrogenase and cleaved caspase 3/7 increased significantly at the highest DA concentration but not at lower DA concentrations. Increased keratin 5 ubiquitination occurred after DA exposure but resolved by day 3. Damage to airway basal cells persisted at day 3 in the presence of MG132.
Conclusions: Diacetyl exposure results in airway basal cell injury with keratin 5 ubiquitination and decreased p63 expression. The ubiquitin-proteasome-pathway partially mediates airway basal cell repair after acute DA exposure.

* Corresponding author at: University of Rochester Medical Center, Dept. of Pediatrics, Div. of Pulmonology, 601 Elmwood Avenue, Box 667, Rochester, NY 14642, United States.
E-mail address: matthew_mcgraw@urmc.rochester.edu (M.D. McGraw).

https://doi.org/10.1016/j.toxlet.2020.02.012
Received 11 December 2019; Received in revised form 15 February 2020; Accepted 25 February 2020
Available online 26 February 2020
0378-4274/

1. Introduction

Diacetyl (DA; 2,3-butanedione) is a flavoring chemical added or naturally occurring in a variety of foods and alcoholic beverages. In the past decade, DA use as a flavoring additive has risen dramatically in foods and e-cigarettes (Allen et al., 2016; Pierce et al., 2014). Though previously classified as safe for consumption by the FDA, DA inhalation is associated with debilitating airways disease in humans known as bronchiolitis obliterans (BO) (Bailey et al., 2015; Kreiss et al., 2002). Despite its common use and limited regulation, the exact mechanism of how exposure to DA vapor causes flavoring-induced lung disease remains poorly understood.

Inhalation exposure to DA vapor can result in severe and debilitating lung disease, known as flavoring-related lung disease. The sentinel report of a cohort of workers employed by a microwave popcorn factory in Missouri, US, occurred in May 2000 (Kreiss et al., 2002; Simoes et al., 2002). Workers presented with shortness of breath and cough. Lung function testing demonstrated severe decreases in forced expiratory volume in 1 sec (FEV1) without response to bronchodilator medication, supportive of severe, fixed obstructive lung disease. Further investigation by the National Institute for Occupational Safety and Health (NIOSH) identified 10-fold greater rate of decline in lung function of exposed workers compared to non-smoking controls (Kreiss et al., 2002). NIOSH extended their evaluation to five additional microwave popcorn plants total greater than 700 workers evaluated for symptoms and lung function. DA-exposed workers, and more specifically mixers, with greater than one year of work had more chest symptoms and poorer lung function as measured by FEV1 than mixers who worked for less than one year (Kanwal et al., 2006). Collectively, these investigations highlight the risk of severe respiratory impairment and debilitating, irreversible lung disease associated with DA inhalation exposure.

Following the sentinel report of BO in popcorn factory workers exposed to DA vapors (Kanwal et al., 2006; Kreiss et al., 2002; Simoes et al., 2002), multiple preclinical *in vivo* models of DA vapor exposure developed. Acutely, rats exposed to DA vapors for 6 h at greater than 100 ppm develop airway epithelial injury (Hubbs et al., 2002, 2016). Sub-acutely, rats exposed to multiple days of DA vapors develop fibrotic, intrapulmonary airway lesions (Morgan et al., 2016). Additionally, when allowed to recover for two weeks after DA exposures, fibrotic airway lesions persist in exposed rats (Morgan et al., 2016). Collectively, these *in vivo* rat models of DA exposure provide evidence of direct airway epithelial injury as well as persistent airway remodeling recapitulating some of the human pathology.

To complement these *in vivo* models of DA vapor exposures, *in vitro* DA vapor exposure models have also developed (Brass et al., 2017; Foster et al., 2017; Kelly et al., 2014; Park et al., 2019). One of the *in vitro* culture systems developed for studying DA vapor exposures utilized primary human bronchial epithelial cells differentiated at air-liquid interface (ALI) (Kelly et al., 2014). When exposed to one-hour of 25 mM DA (~1000 ppm) vapors for repetitive exposures, a significant rise in supernatant lactate dehydrogenase (LDH) occurred (Brass et al., 2017; Kelly et al., 2014). Zaccone et al. also evaluated airway epithelial function and morphometry in an air-liquid interface culture after a six-hour exposure to 25 ppm DA (Zaccone et al., 2015). A significant reduction in sodium transport occurred at 18 h after DA exposure, but without a significant change in trans-epithelial electrical resistance (TEER). At this lower concentration, single day exposure, the airway epithelium also retained its ability to metabolize DA to its less reactive metabolites *via* dicarbonyl/L-xylulose reductase (DCXR). At higher DA concentrations (100 − 360 ppm) for six hours, the airway epithelial layer detached, resulting in cellular death (Zaccone et al., 2015). Thus, at lower DA concentrations or for shorter exposure periods, the airway epithelium retains its ability to recover from chemical exposure, but for longer or higher concentration exposures, DA causes epithelial cell death. Thus, after a single DA exposure, the airway epithelium retains its ability to recover from chemical exposure, but after repeated DA exposures, airway epithelial cell death occurs.

We hypothesize that the airway epithelium retains its regenerative capacity following a single, lower concentration DA vapor exposure through airway basal cell repair. The primary purpose of this study was to evaluate for airway basal cell injury and repair following a single one-hour DA vapor exposure in primary human airway epithelial cultures exposed to clinically relevant concentrations of DA vapors.

2. Materials and methods

2.1. Chemical

Diacetyl (2,3-butanedione, 98.5 % purity) was purchased from Sigma-Aldrich (St. Louis, MO). The proteasome inhibitor MG132 (> 98 % purity) was purchased from Tocris (Pittsburgh, PA).

2.2. Primary human airway epithelial cultures

Human EpiAirway™ (AIR-100) generated from primary human bronchial epithelial cells from healthy, non-smoking donor (TBE-20), were purchased from MatTek, Corporation (Ashland, MA). All tissues were well differentiated at air-liquid interface (ALI) on a microporous (9 mm internal diameter) membrane in plastic inserts prior to exposure. Upon receipt from MatTek, cultures were placed into 1 ml of culture medium (MatTek, Ashland, MA) in 6-well culture plates for at least 24 h to equilibrate prior to exposure.

2.3. In vitro diacetyl (DA) vapor cup exposure

Immediately prior to exposure, DA was diluted in phosphate-buffered solution (PBS) to final concentrations of 12, 25, and 50 mM. PBS vehicle was used a negative control. Concentrations were chosen from previous published estimations of vapor concentrations within the vapor cup exposure at 37 °C, and relevant to peak levels of DA in factories with artificial butter flavoring (Brass et al., 2017; Foster et al., 2017; Kelly et al., 2014; Kreiss et al., 2002). The majority of experiments were conducted at DA exposure concentrations of 25 and 50 mM. These concentrations were chosen to model the intermittent, high-concentration exposure or mixers in buttery factories. Previous publications have calculated the expected DA vapor concentrations to be ~1100 and ~2200 ppm for 25 mM and 50 mM DA exposures (Brass et al., 2017; Kelly et al., 2014). Using equivalent DA concentrations allows for direct comparison to prior publications on *in vitro* DA vapor cup exposures as well as can be used to contrast results of single and multiple DA vapor exposures (Brass et al., 2017; Foster et al., 2017; Kelly et al., 2014).

ALI tissue cultures were exposed to DA-derived vapors for one-hour using vapor cups as described previously (Kelly et al., 2014). Briefly, 50 microliters (μl) DA or PBS was pipetted onto a 6 mm. antibiotic sensitivity disk (BD BBL™; Franklin Lakes, NJ) placed within a 1.5 ml Eppendorf tube top. The vapor cup was inverted over the tissue culture, sealed tightly onto the plastic well insert, and placed into the 5% CO_2 incubator for 1 h. After 1 h, the Eppendorf tube top was removed, and the tissue cultures were returned to the incubator prior to analysis at 1, 3 and 5 days after DA exposure. A single, one-hour DA vapor exposure was used (over repeated exposure) to characterize the concentration and temporal response of airway epithelial basal cells to an acute DA vapor exposure. Ten separate DA exposures occurred with different human samples for each exposure. All DA exposures were performed with four replicates per exposure and repeated at least twice with the same donor for each experiment.

2.4. Lactate dehydrogenase (LDH) activity

Lactate dehydrogenase (LDH) activity (Thermo Scientific Pierce;

Rockford, IL) was measured in apical washes of the cellular supernatant as a surrogate marker of cellular injury after exposure. The apical surface of the ALI tissues was gently rinsed with 0.4 ml PBS prior to exposure and at 23 h after exposure (Day 1). Apical rinses were centrifuged prior to LDH activity testing to remove mucus/debris. LDH activity was expressed as fold change over PBS (vehicle) control.

2.5. Caspase-3/7 activity

As a marker of cellular apoptosis, supernatant caspase-3/7 (Casp-3/7) activity was measured in apical washes of the cellular supernatant. The apical surface of the ALI tissues was gently rinsed with 0.4 ml PBS prior to exposure and at 23 h after exposure (Day 1). Apical rinses were centrifuged prior to testing to remove mucus/debris. After centrifugation, Casp-3/7 activity was generated by following the commercially available protocol as described (Promega, Madison, WI). Luminescent activity was expressed as fold change over PBS (vehicle) control.

2.6. Trans-epithelial electrical resistance (TEER)

Trans-epithelial electrical resistance (TEER) was measured in all tissue cultures prior to and at 1 day after exposure using silver chloride electrodes (EVOM, World Precision Instruments, Sarasota, FL)(Anders, 2017). Electrodes were connected to the volt-ohmmeter and were equilibrated in balanced PBS solution (MatTek) for 15 min before use. 0.4 ml of warm PBS solution (MatTek) was added to the apical surface of ALI cultures. TEER was measured by placing the longer electrode into the basal media, and the shorter electrode into the apical transwell insert. Two measurements were taken from each insert. ALI cultures were not used for exposure if the TEER measurement was ≤ 300 Ohms*cm^2 (Wang et al., 2019; Zaccone et al., 2015)

2.7. Histologic analyses of airway cultures

On Day 1 after exposure, tissue cultures were fixed in 10 % neutral buffered formalin (NBF) overnight at 4 °C followed by cold PBS wash. Tissue cultures were excised from transwell insert, placed in a Kim wipe and enclosed within a tissue embedding cassette. Tissue cultures were dehydrated in 80 % ethanol, embedded in paraffin, sectioned (5 μm), and mounted on silane-coated glass slides. Sections were stained with hematoxylin and eosin (H&E).

2.8. Immunofluorescent staining for basal and ciliated epithelial cell markers

Embedded sections of airway epithelium were stained for common airway epithelial cell markers, including keratin 5 (Krt5; 1:2000, Biolegend, Dedham, MA), ΔN isoform of transcription factor p63 (ΔNp63; 1:200, Biolegend, Deham, MA), Ki-67 staining (1:200, Abcam, Cambridge, MA), and acetylated tubulin (AT, 1:1000, Millipore Sigma, St. Louis, MO). Rabbit and Mouse IgG (1:1000, Agilent, Santa Clara, CA) were used as negative controls. Briefly, sections were deparaffinized with xylene, followed by dehydration in graded alcohol and heated in antigen retrieval solution (Vector, Burlingame, CA). Sections were washed in PBS buffer and blocked with 10 % normal BSA in PBS for 1 h at room temperature. Slides were then rinsed and incubated overnight at 4 °C with respective primary antibody. After PBS rinse, the slides were counterstained with an AlexaFluor secondary immunofluorescent antibody (1:1000; ThermoFisher Scientific, Rockford, Illinois, USA) and mounted with DAPI Fluoromount-G (Southern Biotechnology, Birmingham, Alabama).

2.9. Western blot analyses for Krt5, ΔNp63, β-actin, and GAPDH

Airway cultures were homogenized in RIPA lysis buffer (Abcam; Cambridge, MA) supplemented with a protease inhibitor cocktail (Roche; Indianapolis, IN). Following centrifugation at 12,000 rpm for 20 min at 4 °C, soluble supernatant fractions were collected for total protein and western blot analysis. Total protein concentrations were determined by BCA assay kit (Thermo Scientific, Waltham, MA). Ten micrograms (μg) total protein were resolved in pre-casted 4–15 % gradient Tris-Glycine gel (Bio-Rad, Hercules, CA), and immunoblotted for Krt5 (1:5000; Biolegend), ΔNp63 (1:1000; Biolegend), and K48-ubiquitin (1:1000, R&D Systems). Beta-actin and GAPDH served as loading controls and for densitometry analysis normalization. Gels were transferred to 0.1 μm nitrocellulose membrane (GE Healthcare). HRP and SuperSignal West Pico chemiluminescent substrates (Thermo Scientific) were used to detect protein signal intensity.

2.10. Immunoprecipitation of Krt5 from airway epithelial homogenates

To isolate Krt5 from total airway homogenates, tissue culture lysates were intubated with 50 μl magnetic beads (Miltenyi Biotec) and 2 μl Krt5 antibody (1 mg/ml) overnight. Solution was then passed through a magnetic column. The column was washed multiple times using RIPA buffer (Abcam) and eluted using SDS PAGE (Bio-Rad). The immunoprecipitated protein was quantified *via* Western blot analysis as detailed above. Co-localization of polyubiquitin-C with Krt5 was performed by performing immunoblotting for Ubiquitin C antibody (1:1000, Invitrogen) on immunoprecipitated Krt5 protein. Airway cultures incubated with 100 mM MG132 for 24 h was used as a positive control.

2.11. Proteasome activity of total airway epithelial cellular homogenates

To measure 20S, chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (C–L) proteasome activities in airway cultures, cultures were lysed and collected in 50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl and 1% Triton X-100 supplemented with 2 mM ATP buffered solution. Proteasome activities were determined using the Chemicon 20S Proteasome Activity Assay (Millipore) and Proteasome-Glo 3 Substrate System, respectively, according to the manufacturer's instructions. The luminescent signal was quantified in a SpectraMax M5 plate reader (Molecular Devices, San Jose, CA). Enzymatic activity in DA-exposed samples was expressed relative to the activity of PBS controls.

2.12. Proteasome inhibition of exposed airway epithelial samples with MG132

To address whether proteasome activity is involved with keratin 5 DA-induced ubiquitination and degradation, primary human airway cultures were co-incubated with proteasome inhibitor MG132 (100 nM; Tocris, > 98 % purity). 100 nM MG132 concentration is published previously as a non-cytotoxic concentration in *in vitro* cell cultures of normal human bronchial epithelial cells (Krunkosky et al., 2003). MG132 was diluted in the basolateral maintenance media (MatTek) of the airway cultures and replaced every other day after DA exposure.

2.13. Statistical analysis

For normally distributed data, results of quantitative measures were expressed as means ± standard deviation (SD) and analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* analysis. When data were not normally distributed, results were expressed as medians with min/max error bars and analyzed using a Kruskal-Wallis test. All data were analyzed using with Prism 7.0 software (GraphPad, La Jolla, CA).

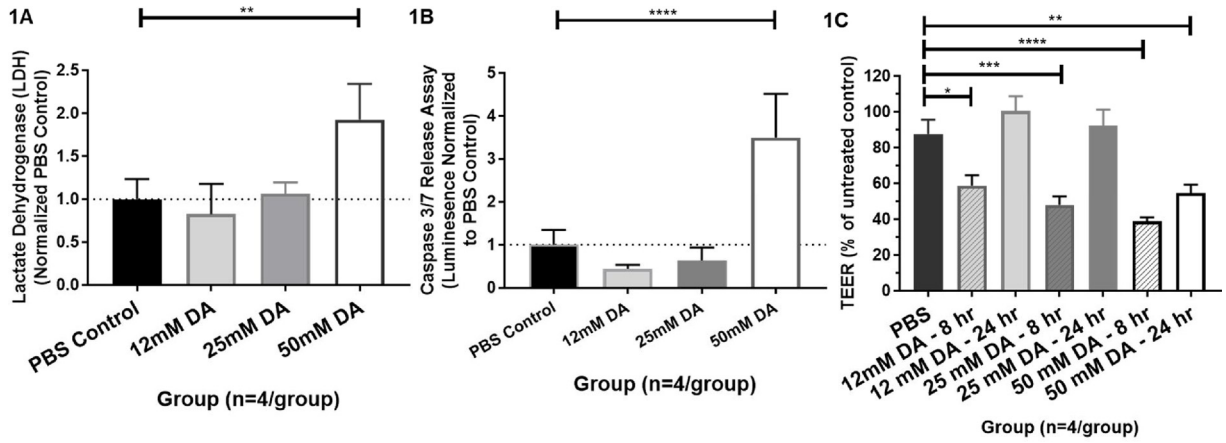


Fig. 1. (A) Supernatant lactate dehydrogenase (LDH) at 24 h post exposure. Diacetyl (DA) concentrations included 12, 25, and 50 mM or PBS control. Supernatant LDH differed significantly from PBS control at 50 mM DA exposure (ANOVA; n = 4/group; **p < 0.01) (B) Supernatant caspase 3/7 release assay at 24 h post exposure. Diacetyl (DA) concentrations included 12, 25, and 50 mM or PBS control. Supernatant caspase 3/7 release differed significantly at 50 mM DA exposure from PBS control (ANOVA; n = 4/group; ****p < 0.0001). (C) Trans-epithelial electrical resistance (TEER) at 8 and 24 h post-DA exposure for 12, 25, and 50 mM DA concentrations. TEER differed significantly at 8 h from PBS controls in all DA concentrations (ANOVA with Tukey's; n = 4/group; 12 mM DA, *p < 0.05; 25 mM DA, ***p < 0.001; 50 mM DA, ****p < 0.0001) and at 24 h at 50 mM DA (ANOVA; n = 4/group; **p < 0.01).

3. Results

3.1. Rise in lactate dehydrogenase (LDH) and cleaved caspase 3/7 activity after a single one-hour DA vapor exposure

Lactate dehydrogenase (LDH) and cleaved caspase 3/7 (Casp-3/7) activity in airway supernatants were used as surrogate markers of cell death and apoptosis after DA exposure, respectively. Twenty-four hours after exposure, airway supernatant LDH increased significantly in 50 mM DA-exposed cultures compared to PBS, 12 and 25 mM DA-exposed culture (Fig. 1A; ANOVA with Tukey's; n = 4/group; ** p < 0.01). Airway supernatant Casp-3/7 also increased significantly in 50 mM DA-exposed cultures compared to PBS, 12 and 25 mM DA-exposed cultures (Fig. 1B; ANOVA; n = 4/group; **** p < 0.0001). Both LDH and Casp-3/7 did not differ in 12 and 25 mM DA-exposed cultures from PBS controls. These results suggest that cell death in human airway epithelial cultures did not occur until 50 mM DA exposure for one hour and is partially mediated through apoptosis.

3.2. Transient decrease of trans-epithelial electrical resistance (TEER)

Trans-epithelial electrical resistance (TEER) was used as a marker of airway epithelial cellular permeability before and after DA exposures. In all DA-exposed cultures, TEER measurements decreased significantly at 8 h post-exposure compared to PBS exposed controls (Fig. 1C; ANOVA; n = 4/group; * p < 0.05). TEER remained significantly decreased at 24 h in 50 mM DA-exposed cultures (ANOVA with Tukey's; n = 4/group; ** p < 0.01), but did not differ significantly at lower DA concentrations (12 and 25 mM) from PBS controls. Thus, all airway epithelial cultures at the tested DA concentrations demonstrated a transient decrease in airway epithelial permeability within 8 h of DA exposure, but only cells exposed to 50 mM DA demonstrated persistent cellular permeability at 24 h.

3.3. H&E of primary human airway epithelial cells exposed to DA

Human airway epithelial cultures were fixed at 24 h post-exposure to evaluate for histologic evidence of airway injury. Airway epithelial cultures exposed to PBS demonstrated no morphologic changes by histology (Fig. 2A). Conversely, morphologic changes to the airway epithelium demonstrated intra- and intercellular clearing with cytoplasmic hypopigmentation in the basal and suprabasal layers (Fig. 2B and C; *white arrows*).

3.4. Antibody staining for common airway epithelial cell markers

Considering DA exposure primarily affected the airway basal cell layer, staining for common airway basal cell markers, specifically Krt5 and delta N p63 (ΔNp63) was performed. Acetylated tubulin, a common marker for airway ciliated cells, was also performed for comparison. Compared to PBS controls, more prominent Krt5 staining was apparent in 25 mM and 50 mM DA-exposed culture sections (Fig. 2D–F; *green; solid arrows*). Staining for ΔNp63 decreased in 50 mM DA-exposed cultures (Fig. 2G–I; *brown*) compared to PBS controls. Ciliated cell staining with acetylated tubulin did not differ between PBS controls and DA-exposed cultures (Fig. 2D–F; *red; hollow arrows*). Considering increased expression of Krt5 may be due to increased basal cell proliferation, Ki67+ staining was also performed in PBS and DA-exposed cultures. The number of Ki67+ cells per 1000 μm of culture basement membrane did not differ significantly between 25 mM DA exposed sections (Supplemental Figure A.1; n = 8/group; t-test, p = 0.20).

3.5. Quantification of airway basal cell markers - Krt5 and ΔNp63

To quantitate changes seen on histology, western blot analyses for Krt5, ΔNp63, and β-actin (internal control) were performed. Krt5 expression (58 kDa) normalized for β-actin did not differ significantly in 25 mM and 50 mM DA-exposed cultures compared PBS controls (Fig. 3A; n = 4/group; ANOVA, p > 0.05). Surprisingly, a significant increase in Krt5 expression was seen at higher molecular weights in both 25 and 50 mM DA-exposed cultures (Fig. 3A; n = 4/group; ANOVA, **p = 0.01). Quantification of Krt5 expression at 116 kDa (double Krt5 molecular weight) normalized for β-actin differed significantly at 24 h in 25 mM DA (Fig. 3A; n = 4/group; ANOVA, ***p < 0.001) and 50 mM DA (Fig. 3A; n = 4/group; ANOVA with Tukey's, **p < 0.01) cultures compared to PBS controls. ΔNp63α decreased significantly in 50 mM DA-exposed cultures compared to PBS controls (Fig. 3B; n = 4/group; ANOVA, *p = 0.016).

3.6. Time-dependent degradation of keratin 5 after DA exposure

Next, we assessed for changes to Krt5 and ΔNp63 expression at 1, 3, and 5 days after 25 mM DA exposure. Increased expression of higher molecular weight Krt5 (116 kDa) occurred in airway homogenates exposed to 25 mM DA vapor compared to PBS controls at Day 1 after DA exposure (left four lanes; Fig. 4A). At Day 3 and Day 5 post-exposure, higher molecular weight Krt5 expression was not significantly increased

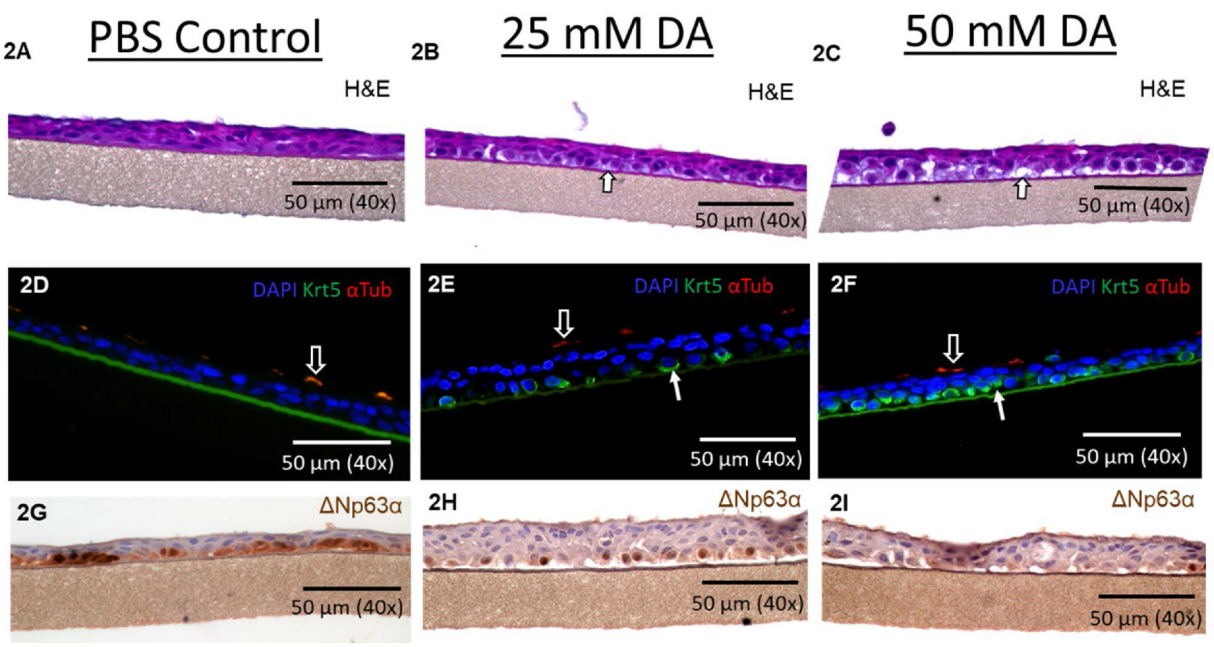


Fig. 2. Representative images of hematoxylin and eosin (H&E)-stained sections of airway epithelial cultures at 24 h after exposure to PBS (A), 25 mM DA (B) and 50 mM DA (C). Representative images of immunofluorescent-stained sections for keratin 5 (Krt5; *green; solid arrow*), ciliated cell (α-tubulin; *red; outlined arrow*), and nuclear (DAPI; *blue*) at 24 h following exposure to PBS (D), 25 mM DA (E) and 50 mM DA (F). Of note, autofluorescence of culture membrane occurred with red and green fluorescent antibodies in PBS controls (D). Representative images of delta N p63 (ΔNp63)-stained sections in airway cultures at 24 h after exposure to PBS (G), 25 mM DA (H) and 50 mM DA (I).

in 25 mM DA-exposed cultures compared to PBS controls (Fig. 4A; n = 4/group; ANOVA with Tukey's, $p > 0.05$). In contrast, Krt5 (58 kDa) decreased at Day 3 and 5 after DA exposure in 25 mM DA-exposed cultures compared to PBS controls (Fig. 4A; n = 4/group; ANOVA, **p = 0.002). ΔNp63 expression also decreased at Day 3 and 5 after DA exposure in 25 mM DA-exposed cultures compared to PBS controls (Fig. 4B; n = 4/group; ANOVA, *p = 0.011).

3.7. Increased K48-ubiquitination and Co-localization of ubiquitin C with Krt5 after exposure

One potential reason for the shift in Krt5 molecular weight seen

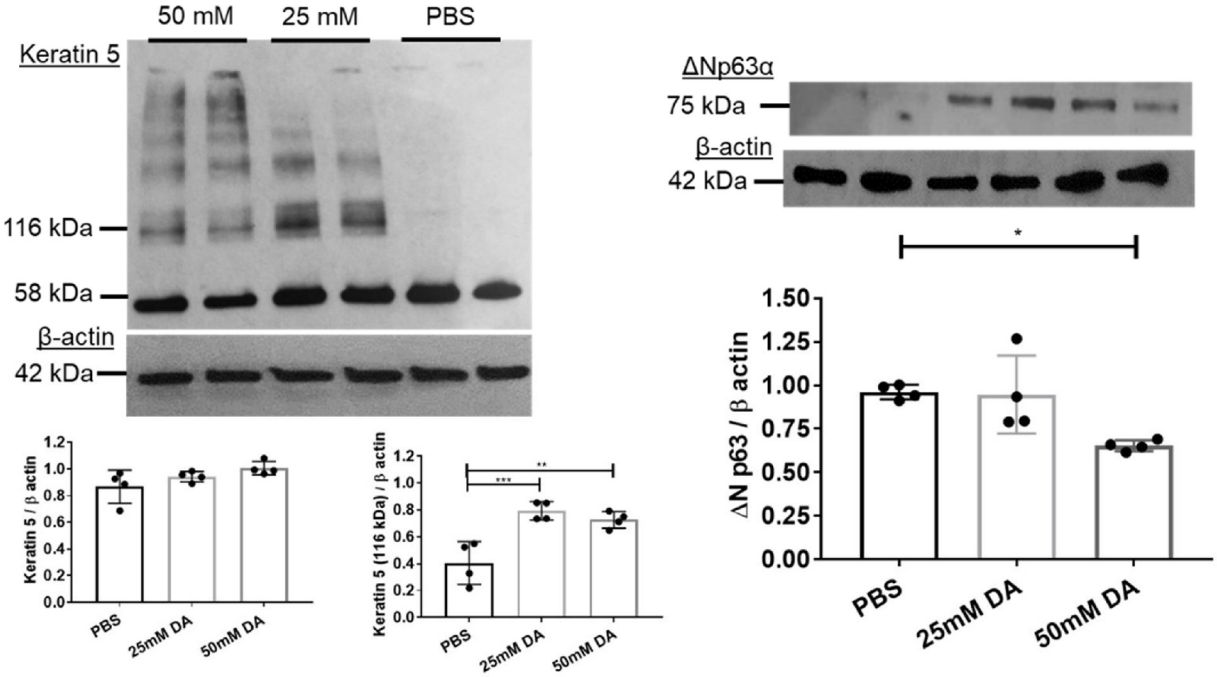


Fig. 3. Representative western blot analyses for (A) Keratin 5 and (B) ΔNp63 expression in airway cellular homogenates at 24 h post-exposure in DA (25 and 50 mM) and PBS controls. Beta-actin used as a loading control (42 kDa). Quantification of Krt5 expression at 58 kDa and 116 kDa normalized to β-actin for PBS, 25 mM, and 50 mM DA (n = 4/group; *lower left graph*). Normalized Krt5 expression at 116 kDa differed significantly from PBS control at 25 mM DA (ANOVA with Tukey's; n = 4/group ***$p < 0.001$; *lower right graph*) and 50 mM DA (ANOVA with Tukey's; n = 4/group; **$p < 0.01$). (B) Normalized ΔNp63α expression decreased significantly from PBS control at 50 mM DA (ANOVA with Tukey's; n = 4/group; *p = 0.016).

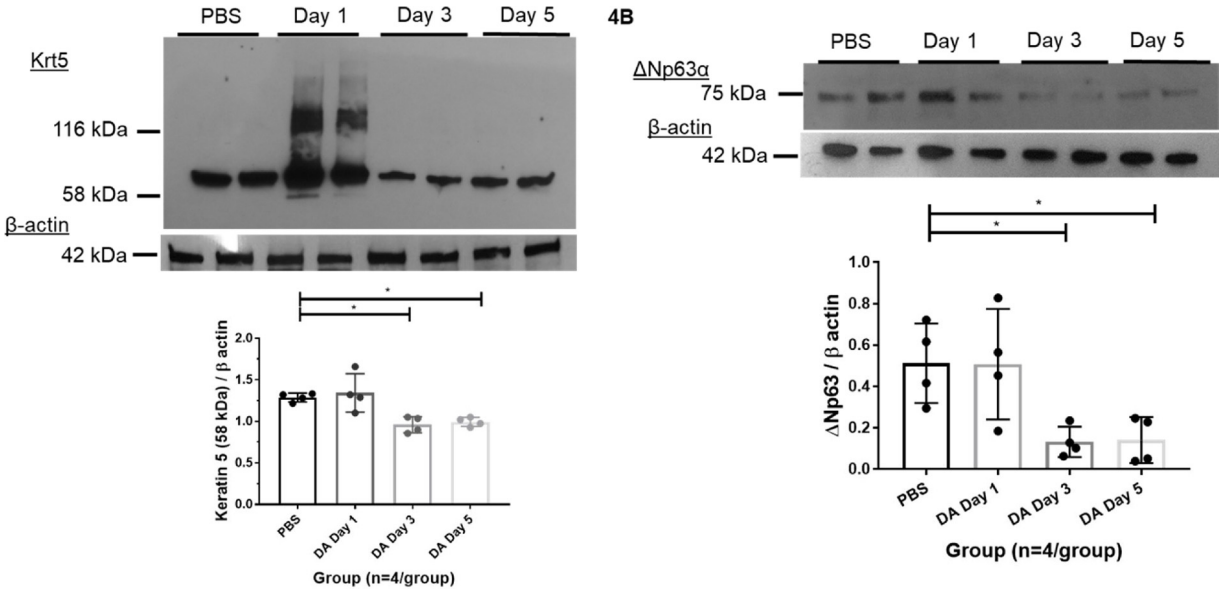


Fig. 4. Representative western blot of (A) keratin 5 (Krt5) and (B) ΔNp63α expression in cellular homogenates exposed to 25 mM DA for 1 h at Day 1, 3 and 5 after exposure compared to PBS controls (n = 4/group). Beta-actin used as a loading control (42 kDa). Normalized Krt5 expression at 58 kDa differed significantly from PBS control at Day 3 (ANOVA with Tukey's; n = 4/group; *p = 0.019) and Day 5 (ANOVA with Tukey's; n = 4/group; **p = 0.035). (B) Normalized ΔNp63α expression decreased significantly from PBS control at Day 3 (ANOVA with Tukey's; n = 4/group; *p = 0.045) and Day 5 (ANOVA with Tukey's; n = 4/group; *p = 0.048).

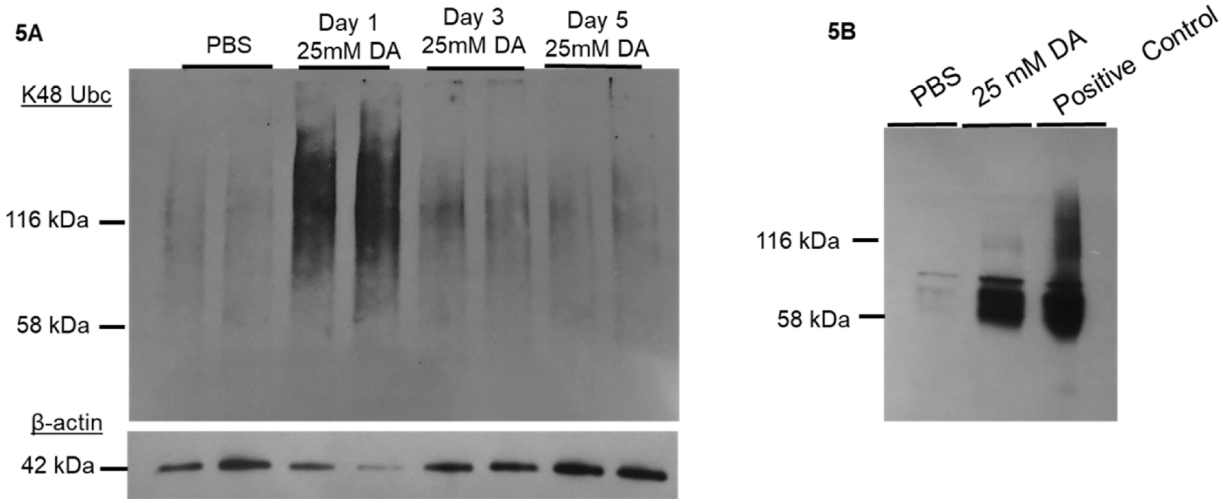


Fig. 5. (A) Representative western blot image of K48-linked ubiquitin in airway cellular homogenates exposed to PBS or 25 mM DA. Beta-actin used as a loading control (42 kDa). Increased staining for K48-linked ubiquitin occurred at Day 1 following 25 mM DA exposure compared to PBS controls (*middle left two lanes*). By Day 3 and 5 after 25 mM DA exposure, K48-linked ubiquitin normalized to PBS control levels. (B) Representative western blot image of ubiquitin C in cellular homogenates immunoprecipitated (IP) for keratin 5 (Krt5) after exposure to PBS or 25 mM DA. Airway cultures incubated with 100 mM MG132 for 24 h were used as a positive control. Increased staining for ubiquitin C in 25 mM DA-exposed Krt5 IP compared to PBS controls.

after DA vapor exposure is keratin ubiquitination due to protein damage (Foster et al., 2017; Rogel et al., 2010). Considering that cytoskeletal keratin ubiquitination requires proteasome degradation for proper recycling (Rogel et al., 2010), we performed staining for K48-linked ubiquitination in exposed human airway epithelial culture homogenates after DA exposure. Airway epithelial cultures exposed to 25 mM DA vapor demonstrated increased staining for K48-linked ubiquitination at Day 1 after DA exposure compared to PBS control samples (Fig. 5A; *middle two lanes*). At three and five days after DA exposure (when Krt5 damage had resolved), K48-linked ubiquitination expression in 25 mM DA-exposed cultures was similar to PBS controls (Fig. 5A; *far right four lanes*).

Next, we performed immunoprecipitation (IP) for Krt5 from human airway epithelial cultures following exposure to 25 mM DA or PBS to assess for co-localization of poly-ubiquitination with Krt5. Airway cultures treated with a high concentration of MG132 (100 mM) but not exposed to DA was used as the positive control (Fig. 5B; *far right lane*). At Day 1, Krt5 IP demonstrated increased ubiquitin C expression in 25 mM DA exposed airway epithelial cultures compared PBS controls samples (Fig. 5B).

3.8. Proteasome activity associated temporally with keratin 5 damage resolution

When K48-linked ubiquitination occurs, proteasome degradation ensues (Jaitovich et al., 2008). Thus, we assessed for proteasome 20S activity following DA exposure in primary human airway epithelial cellular homogenates. No significant change in proteasome 20S activity

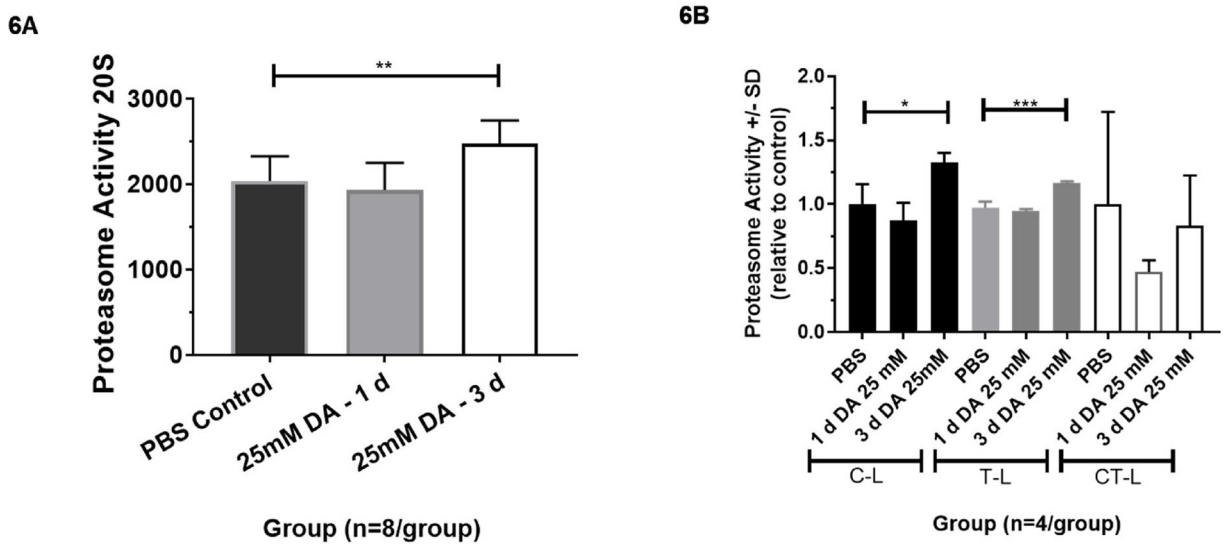


Fig. 6. (A) Proteasome 20S activity in 25 mM DA or PBS vapor-exposed airway epithelial cellular homogenates at 1 and 3 days post-exposure. Proteasome 20S activity increased significantly in 25 mM DA exposed cellular homogenates at Day 3 from PBS control (ANOVA with Tukey's; $^{**}p < 0.01$, n = 8/group). (B) Proteasome activity by sub-type (caspase-like (C−L), trypsin-like (TL−), chymotrypsin-like (CT−L)) in PBS or 25 mM DA exposed samples at 1 and 3 days post-exposure. C−L and TL− activity increased significant in human airway epithelial samples at Day 3 after 25 mM DA exposure compared to PBS and Day 1 DA-exposed samples (ANOVA; n = 4/group; $^{*}p < 0.05$ and $^{***}p < 0.001$, respectively). Conversely, no significant increase in CT−L activity occurred at Day 1 or Day 3 after exposure compared to PBS controls (ANOVA; n = 4/group; $p < 0.05$).

was seen in DA-exposed cultures compared to PBS control samples at Day 1 after DA exposure (Fig. 6A; ANOVA with Tukey's; $p > 0.05$; n = 8/group). By Day 3, proteasome 20S activity increased significantly in DA-exposed controls compared to PBS controls (Fig. 6A; ANOVA with Tukey's; $^{**}p < 0.01$; n = 8/group). Increased proteasome 20S activity correlated temporally (at Day 3) with resolution of higher molecular weight Krt5 expression after DA exposure.

Individual proteolytic site activities, including caspase-like (C–L), trypsin-like (TL−), and chymotrypsin-like (CT−L), were also assessed in cellular homogenates at Days 1 and 3 after DA exposure. Again, no significant change in C–L, TL, or CTL−− activity occurred in DA-exposed cultures compared to PBS controls at Day 1 after exposure (Fig. 6B; n = 4/group; ANOVA with Tukey's, $p > 0.05$). At Day 3 post-exposure, a significant increase in C–L and TL− activity occurred in DA-exposed cultures compared to PBS controls (Fig. 6B; ANOVA with Tukey's; $^{*}p < 0.05$ and $^{***}p < 0.001$, n = 4/group, respectively). No significant increase in CT−L activity in DA-exposed cultures compared to PBS controls occurred at Day 3 after DA exposure (Fig. 6B; n = 4/group; ANOVA with Tukey's, $p > 0.05$). Variance of CT−L activity was greater than that of C–L and TL− activities, most likely due to difference in substrate content, cleavage site preference, and cell type specificity (Kisselev et al., 2006).

3.9. Proteasome inhibition with MG132 causes persistence of keratin 5 injury

To further validate Krt5 protein degradation is mediated through the ubiquitin-proteasome pathway after DA exposure, human primary airway epithelial cells were incubated with the reversible proteasome inhibitor MG132 (100 nM) daily for 3 days. Prior to DA exposure, we verified proteasome 20S activity being significantly inhibited in PBS controls incubated with MG132 compared to PBS controls without MG132 (Supplemental Figure A.2; Welch's t-test; n = 4/group; $^{**}p < 0.0011$). Similar to prior 25 mM DA exposures, increased Krt5 expression at higher molecular weight (116 kDa) occurred at Day 1 after DA exposure with and without MG132 co-incubation (Fig. 7A; *middle 8 lanes*; n = 4/group; ANOVA, $^{****}p < 0.0001$). In contrast, higher molecular weight Krt5 expression persisted at Day 3 after DA exposure when co-incubated with MG132, but resolved in the absence of MG132 (Fig. 7A; *far right eight lanes*). Higher molecular weight Krt5 expression differed significant in 25 mM DA-exposed cultures + MG132 compared to 25 mM DA-exposed cultures alone (Fig. 7B; n = 4/group; ANOVA with Tukey's, $^{*}p = 0.04$). Co-incubation with MG132 also increased Krt5 expression at 58 kDa at Day 3 after 25 mM DA exposure compared to Day 3 25 mM DA without MG132 (Fig. 7B; n = 4/group; ANOVA with Tukey's, $^{***}p = 0.0002$). Collectively, proteasome inhibition with MG132 suggest impaired clearance of Krt5 following DA vapor exposure.

4. Discussion

A single, one-hour diacetyl (DA) vapor exposure caused significant airway basal cell injury with ubiquitination of keratin 5 (Krt5) and decreased ΔNp63. At DA exposure concentrations below 50 mM DA for one hour, the airway epithelium recovered without significant cellular death or persistent loss of cellular integrity by trans-epithelial electrical resistance. Independent of cell death or loss of barrier integrity, DA exposure resulted in histologic evidence of basal cell injury with decreased ΔNp63 expression and Krt5 ubiquitination. When monitored for up to five days after exposure, ubiquitin-proteasome system activation occurred, resulting in the subsequent reduction of total Krt5 and ΔNp63 expression. Additionally, proteasome inhibition resulted in the persistence of DA-induced damage to basal cell keratin 5.

Unique to this work, changes to the airway basal cells occur prior to a significant rise in lactate dehydrogenase after a single DA exposure. Common airway basal cell markers, specifically cytoplasmic keratin 5 and transcription factor ΔNp63α, were used for identification of DA-induced basal cell injury after exposure. Using immunoprecipitation, poly-ubiquitination co-localized with Krt5 following DA exposure (Fig. 5B). In prior experiments using repeated DA exposures, other investigators have also identified damage to airway keratins (Foster et al., 2017). One potential mechanism for this damage to airway keratins is non-enzymatic protein adduction of DA to arginine (Mathews et al., 2010). Arginine is found abundantly on keratin intermediate filament (Anders, 2017). Protein adduction occurs through a Michael reaction, where DA (an electrophile) reacts non-enzymatically with arginine (a nucleophile). We hypothesize the increased susceptibility of airway basal cells to DA vapor exposure is secondary to the high reactivity of

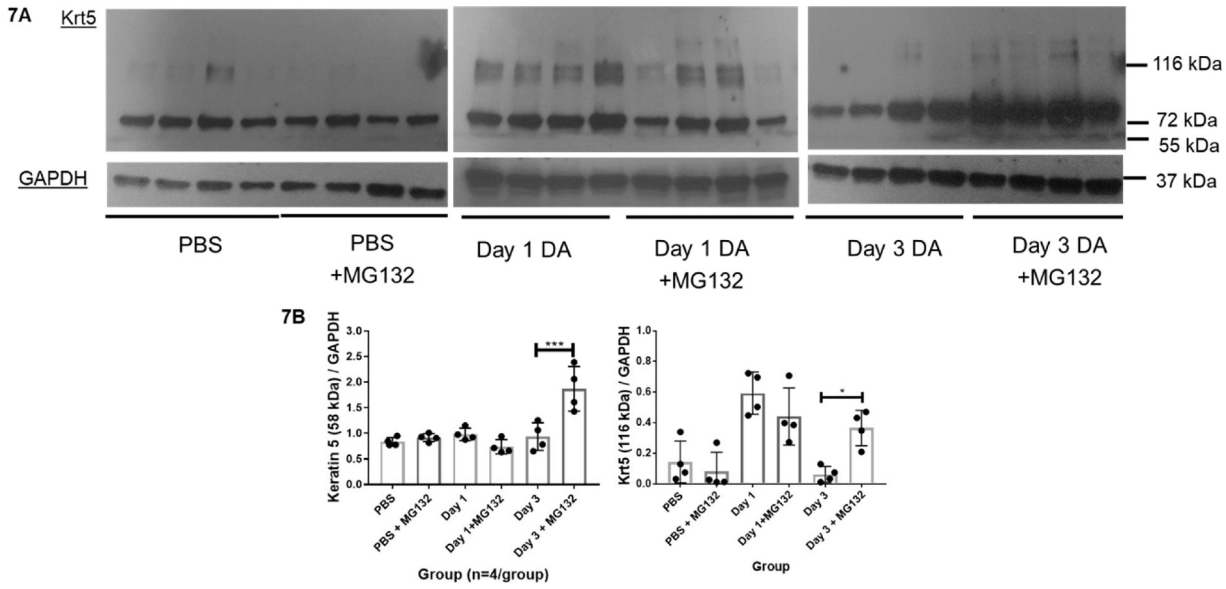


Fig. 7. (A) Representative western blot images of Keratin 5 in PBS or 25 mM DA-exposed airway epithelial cells co-incubated with proteasome inhibitor MG132 (100 nM) or PBS for 1 or 3 days after exposure. GAPDH expression used as a loading control. (B) Quantification of Keratin 5 (58 kDa; *left*) and higher molecular weight Krt5 (116 kDa; *right*) normalized to GAPDH under each condition (n = 4/group). Increased expression of Krt5 (58 kDa) in cellular homogenates at Day 3 after exposure to 25 mM DA exposure + MG132 (100 nM) compared to Day 3 25 mM DA without MG132 (ANOVA; n = 4/group, ***p = 0.0002). When treated with MG132 100 nM, normalized Krt5 at 116 kDa persisted in cellular homogenates at 3 days after DA 25 mM exposure with increased expression compared to Day 3 25 mM DA without MG132 (ANOVA with Tukey's; n = 4/group *p = 0.043).

DA with the common and abundant nucleophilic base arginine on keratin intermediate filaments. Our work emphasizes the specific susceptibility of airway basal cells to DA vapor exposure.

In addition to Krt5 ubiquitination, transcription factor ΔNp63α expression decreased after a single 50 mM DA exposure. One of the primary functions of p63 is maintenance of epidermal stratification (Koster et al., 2004; Koster and Roop, 2004; Yang et al., 1998). When p63 expression is downregulated, p53 is activated for terminal differentiation, preventing further proliferation (Yang et al., 1998). Two potential long-term effects of decreased ΔNp63 expression after DA exposure are suppressed proliferative capacity and/or early terminal differentiation in airway basal cells. Consistent with the prior, we did not find a significant increase in Ki67+ staining in airway cultures 24 h after DA vapor exposure (Supplemental Figure A.1). Foster et al. identified previously increased involucrin expression, a marker of squamous metaplasia and decreased cilia expression after repeated DA exposure (Foster et al., 2017). Collectively, these results of decreased ΔNp63α with increased involucrin expression are most consistent with early terminal differentiation of the airway basal cells after DA exposure.

The primary purpose of our experiments was to evaluate airway basal cell repair, and not epithelial injury alone, after DA exposure. To characterize the temporal resolution after DA exposure, we evaluated airway epithelial homogenates for changes in Krt5 and ΔNp63α expression at various time points (1, 3 and 5 days) after a single 25 mM DA exposure. Twenty-five millimolar DA was chosen as cells did not undergo cell death or a persistent loss of barrier integrity at this concentration (Fig. 1). Three days after exposure, Krt5 damage resolved in airway cellular homogenates (Fig. 4). Damage resolution occurred temporally (at day 3) with a subsequent decrease in Krt5 and ΔNp63α expression (Fig. 4). When proteasome function was inhibited with MG132, Krt5 damage persisted in the airway epithelial cells (Fig. 7). Proteasome-mediated degradation of epithelial intermediate filaments is the primary damage repair response to stress in the lung, as seen previously with shear stress or hypoxia (Na et al., 2010; Rogel et al., 2010). Under continual shear stress, ubiquitin co-localizes with keratin 8 and 18 (Jaitovich et al., 2008). With the addition of proteasome inhibition, damaged keratins accumulate and form aggresomes (Loeb and Haas, 1994). Similar to shear stress, keratin injury following chemical DA vapor exposure resulted in keratin 5 ubiquitination. To the best of our knowledge, this is the first study to identify keratin 5 ubiquitination and proteasome degradation mediating changes to Krt5 expression after diacetyl vapor exposure.

There are some limitations to the current study requiring further investigation. First, all of the work occurred *in vitro*. Future *in vivo* studies are required to evaluate the implications of decreased ΔNp63 expression and/or Krt5 ubiquitination after DA exposure. Second, changes to the airway epithelium were characterized after a single DA exposure. The calculated parts-per-million (ppm) concentrations for the DA exposures ranged from 500 to 2000 ppm (Brass et al., 2017; Kelly et al., 2014). These concentrations are similar in magnitude to peak DA concentrations measured in popcorn plant factories (Boylstein et al., 2006; Kanwal et al., 2006) as well as those concentrations used in previous *in vitro* airway epithelial culture experiments (Kreiss et al., 2002). Future experiments are required to assess whether increased keratin ubiquitination contributes to decreased p63 expression or additional basal cell dysfunction after DA exposure. Lastly, we did not assess for other non-proteasome causes of DA-induced epithelial injury. Other non-proteasome signaling cascades, such as K63-linked ubiquitination, may be activated after DA exposure (Hubbs et al., 2016).

In conclusion, a single one-hour DA vapor exposure causes significant airway basal cell injury with keratin 5 ubiquitination and decreased delta Np63 expression. With sufficient time, keratin 5 ubiquitination resolves, and is mediated through proteasome degradation. Future studies are required to evaluate the persistent effects of keratin 5 damage on airway basal cell function after repeated *in vitro* and *in vivo* DA vapor exposures.

Funding

This work was supported in part by the Department of Pediatrics at the University of Rochester through the David H. Smith Fund, the Western New York (WNY) Center for Research on Flavored Tobacco Products (CRoFT) supported by a grant from the National Cancer

Institute (NCI) (5U54CA228110), and the University of Rochester Environmental Health Sciences Center (EHSC), an NIH/NIEHS-funded program (P30 ES001247).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the University of Rochester's Inhalation Exposure Facility, specifically Director Alison Elder, PhD, David Chalupa, MS, and Robert Gelein, MS, for their continued support with diacetyl inhalation exposures. Authors also thank Antony Leonard, MS, for his assistance with immunoprecipitation and Jon Oldach and Anna Maione with MatTek, Corporation for support on the project.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2020.02.012.

References

Allen, J.G., Flanigan, S.S., LeBlanc, M., Vallarino, J., MacNaughton, P., Stewart, J.H., Christiani, D.C., 2016. Flavoring Chemicals in E-Cigarettes: Diacetyl, 2,3-Pentanedione, and Acetoin in a Sample of 51 Products, Including Fruit-, Candy-, and Cocktail-Flavored E-Cigarettes. Environ. Health Perspect. 124, 733–739.

Anders, M.W., 2017. Diacetyl and related flavorant alpha-Diketones: Biotransformation, cellular interactions, and respiratory-tract toxicity. Toxicology 388, 21–29.

Bailey, R.L., Cox-Ganser, J.M., Duling, M.G., LeBouf, R.F., Martin Jr., S.B., Bledsoe, T.A., Green, B.J., Kreiss, K., 2015. Respiratory morbidity in a coffee processing workplace with sentinel obliterative bronchiolitis cases. Am. J. Ind. Med. 58, 1235–1245.

Boylstein, R., Piacitelli, C., Grote, A., Kanwal, R., Kullman, G., Kreiss, K., 2006. Diacetyl emissions and airborne dust from butter flavorings used in microwave popcorn production. J. Occup. Environ. Hyg. 3, 530–535.

Brass, D.M., Gwinn, W.M., Valente, A.M., Kelly, F.L., Brinkley, C.D., Nagler, A.E., Moseley, M.A., Morgan, D.L., Palmer, S.M., Foster, M.W., 2017. The Diacetyl-Exposed Human Airway Epithelial Secretome: New Insights into Flavoring-Induced Airways Disease. Am. J. Respir. Cell Mol. Biol. 56, 784–795.

Foster, M.W., Gwinn, W.M., Kelly, F.L., Brass, D.M., Valente, A.M., Moseley, M.A., Thompson, J.W., Morgan, D.L., Palmer, S.M., 2017. Proteomic analysis of primary human airway epithelial cells exposed to the respiratory toxicant diacetyl. J. Proteome Res. 16, 538–549.

Hubbs, A.F., Battelli, L.A., Goldsmith, W.T., Porter, D.W., Frazer, D., Friend, S., Schwegler-Berry, D., Mercer, R.R., Reynolds, J.S., Grote, A., Castranova, V., Kullman, G., Fedan, J.S., Dowdy, J., Jones, W.G., 2002. Necrosis of nasal and airway epithelium in rats inhaling vapors of artificial butter flavoring. Toxicol Appl Pharm 185, 128–135.

Hubbs, A.F., Fluharty, K.L., Edwards, R.J., Barnabei, J.L., Grantham, J.T., Palmer, S.M., Kelly, F., Sargent, L.M., Reynolds, S.H., Mercer, R.R., Goravanahally, M.P., Kashon, M.L., Honaker, J.C., Jackson, M.C., Cumpston, A.M., Goldsmith, W.T., McKinney, W., Fedan, J.S., Battelli, L.A., Munro, T., Bucklew-Moyers, W., McKinstry, K., Schwegler-Berry, D., Friend, S., Knepp, A.K., Smith, S.L., Sriram, K., 2016. Accumulation of ubiquitin and Sequestosome-1 implicate protein damage in diacetyl-induced cytotoxicity. Am. J. Pathol. 186, 2887–2908.

Jaitovich, A., Mehta, S., Na, N., Ciechanover, A., Goldman, R.D., Ridge, K.M., 2008. Ubiquitin-proteasome-mediated degradation of keratin intermediate filaments in mechanically stimulated A549 cells. J. Biol. Chem. 283, 25348–25355.

Kanwal, R., Kullman, G., Piacitelli, C., Boylstein, R., Sahakian, N., Martin, S., Fedan, K., Kreiss, K., 2006. Evaluation of flavorings-related lung disease risk at six microwave popcorn plants. J. Occup. Environ. Med. 48, 149–157.

Kelly, F.L., Sun, J., Fischer, B.M., Voynow, J.A., Kummarapurugu, A.B., Zhang, H.L., Nugent, J.L., Beasley, R.F., Martinu, T., Gwinn, W.M., Morgan, D.L., Palmer, S.M., 2014. Diacetyl induces amphiregulin shedding in pulmonary epithelial cells and in experimental bronchiolitis obliterans. Am. J. Respir. Cell Mol. Biol. 51, 568–574.

Kisselev, A.F., Callard, A., Goldberg, A.L., 2006. Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate. J. Biol. Chem. 281, 8582–8590.

Koster, M.I., Roop, D.R., 2004. The role of p63 in development and differentiation of the epidermis. J. Dermatol. Sci. 34, 3–9.

Koster, M.I., Kim, S., Mills, A.A., DeMayo, F.J., Roop, D.R., 2004. p63 is the molecular switch for initiation of an epithelial stratification program. Genes Dev. 18, 126–131.

Kreiss, K., Gomaa, A., Kullman, G., Fedan, K., Simoes, E.J., Enright, P.L., 2002. Clinical bronchiolitis obliterans in workers at a microwave-popcorn plant. N. Engl. J. Med. 347, 330–338.

Krunkosky, T.M., Martin, L.D., Fischer, B.M., Voynow, J.A., Adler, K.B., 2003. Effects of TNFalpha on expression of ICAM-1 in human airway epithelial cells in vitro: oxidant-mediated pathways and transcription factors. Free Radic. Biol. Med. 35, 1158–1167.

Loeb, K.R., Haas, A.L., 1994. Conjugates of ubiquitin cross-reactive protein distribute in a cytoskeletal pattern. Mol. Cell. Biol. 14, 8408–8419.

Mathews, J.M., Watson, S.L., Snyder, R.W., Burgess, J.P., Morgan, D.L., 2010. Reaction of the butter flavorant diacetyl (2,3-butanedione) with N-alpha-acetylarginine: a model for epitope formation with pulmonary proteins in the etiology of obliterative bronchiolitis. J. Agric. Food Chem. 58, 12761–12768.

Morgan, D.L., Jokinen, M.P., Johnson, C.L., Price, H.C., Gwinn, W.M., Bousquet, R.W., Flake, G.P., 2016. Chemical Reactivity and Respiratory Toxicity of the alpha-Diketone Flavoring Agents: 2,3-Butanedione, 2,3-Pentanedione, and 2,3-Hexanedione. Toxicol. Pathol. 44, 763–783.

Na, N., Chandel, N.S., Litvan, J., Ridge, K.M., 2010. Mitochondrial reactive oxygen species are required for hypoxia-induced degradation of keratin intermediate filaments. FASEB J. 24, 799–809.

Park, H.R., O'Sullivan, M., Vallarino, J., Shumyatcher, M., Himes, B.E., Park, J.A., Christiani, D.C., Allen, J., Lu, Q., 2019. Transcriptomic response of primary human airway epithelial cells to flavoring chemicals in electronic cigarettes. Sci. Rep. 9, 1400.

Pierce, J.S., Abelmann, A., Spicer, L.J., Adams, R.E., Finley, B.L., 2014. Diacetyl and 2,3-pentanedione exposures associated with cigarette smoking: implications for risk assessment of food and flavoring workers. Crit. Rev. Toxicol. 44, 420–435.

Rogel, M.R., Jaitovich, A., Ridge, K.M., 2010. The role of the ubiquitin proteasome pathway in keratin intermediate filament protein degradation. Proc. Am. Thorac. Soc. 7, 71–76.

Simoes, E., Phillips, P., Maley, R., Kreiss, K., Malone, J., Kanwal, R., 2002. Fixed obstructive lung disease in workers at a microwave popcorn factory - Missouri 2000-2002 (Reprinted from MMWR, vol 51, pg 345-347, 2002). Jama-J Am Med Assoc 287, 2939–2940 (2002).

Wang, Q., Bhattacharya, S., Mereness, J.A., Anderson, C., Lillis, J.A., Misra, R.S., Romas, S., Huyck, H., Howell, A., Bandyopadhyay, G., Donlon, K., Myers, J.R., Ashton, J., Pryhuber, G.S., Mariani, T.J., 2019. A novel in vitro model of primary human pediatric lung epithelial cells. Pediatr. Res.

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M.D., Dotsch, V., Andrews, N.C., Caput, D., 1998. and McKeon F. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol. Cell 2, 305–316.

Zaccone, E.J., Goldsmith, W.T., Shimko, M.J., Wells, J.R., Schwegler-Berry, D., Willard, P.A., Case, S.L., Thompson, J.A., Fedan, J.S., 2015. Diacetyl and 2,3-pentanedione exposure of human cultured airway epithelial cells: Ion transport effects and metabolism of butter flavoring agents. Toxicol. Appl. Pharmacol. 289, 542–549.