

Popcorn worker's lung: In vitro exposure to diacetyl, an ingredient in microwave popcorn butter flavoring, increases reactivity to methacholine[☆]

J.S. Fedan^{a,*}, J.A. Dowdy^a, K.B. Fedan^b, A.F. Hubbs^a

^a Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV 26505-2888, USA

^b Field Studies Branch, Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, WV 26505, USA

Received 17 November 2005; revised 2 February 2006; accepted 3 February 2006

Available online 20 March 2006

Abstract

Workers who inhale microwave popcorn butter flavorings experience decrements in lung function and can develop clinical bronchiolitis obliterans, i.e., “popcorn worker’s lung” (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). In a rat inhalation model, vapors of an artificial butter flavoring damaged the epithelium of the upper and lower airways (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. Pharmacol.* 185, 128–135). Diacetyl, a butter flavoring component, is a major volatile ketone in the popcorn-processing workplace. We investigated the effects of diacetyl on epithelium of guinea pig isolated airway preparations and the effects of diacetyl in vitro on reactivity to bronchoactive agents. In the isolated, perfused trachea preparation, diacetyl added to the intraluminal (mucosal) bath elicited responses that began with contraction (threshold ca. 3 mM) and ended with relaxation. After a 4-h incubation with intraluminal diacetyl (3 mM), contractions to extraluminal (serosal) methacholine (MCh) were slightly increased; however, sensitivity to intraluminally (mucosally) applied MCh was increased by 10-fold. Relaxation responses of MCh (3×10^{-7} M)-contracted tracheas to extraluminally applied terbutaline and intraluminally applied 120 mM KCl, to evoke epithelium-derived relaxing factor release, were unaffected by diacetyl. Exposure of the tracheal epithelium in Ussing chambers to diacetyl decreased transepithelial potential difference and resistance. These findings suggest that diacetyl exposure compromised epithelial barrier function, leading to hyperreactivity to mucosally applied MCh. The respiratory epithelium appears to serve as an initial target for the toxic effects of diacetyl in the airways.

Published by Elsevier Inc.

Keywords: Airway reactivity; Diacetyl; Bioelectric properties; Epithelium; Guinea pig; Methacholine; Perfused trachea; Popcorn worker’s lung; Terbutaline; Trachea

Introduction

Bronchiolitis obliterans is a progressive obstructive disease of small airways which involves bronchiolar inflammation and epithelial necrosis; ordinarily, it is associated with lung transplantation (Shaw et al., 2002; Laohaburanakit et al., 2003; Chan and Allen, 2004; Scott et al., 2005). However, it may be triggered as a result of adverse drug reactions or inhalation of airborne toxicants (Laohaburanakit et al., 2003). An outbreak of clinical

bronchiolitis obliterans occurred in food industry workers inhaling microwave popcorn artificial butter flavoring (Kreiss et al., 2002). The malady has been termed “popcorn worker’s lung” (Schachter, 2002). Industrial hygiene evaluation of the work site air revealed the presence of more than 100 volatile compounds; present in the highest amounts were diacetyl (most abundant), methyl ethyl ketone, acetoin, 2-nanonone, and acetic acid (Hubbs et al., 2002; Kullman et al., 2005). The levels of volatile flavorings varied with work area, but workers in the mixing area received the highest exposures, varying between an average of 32 ppm diacetyl in 10 area samples and a maximum of 98 ppm. Workers who mix the flavoring ingredients in tanks were potentially exposed to a high level of diacetyl, i.e., 1230 ppm, in the air of a holding tank which is opened during processing (Kreiss et al., 2002).

[☆] The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health. Mention of brand name does not constitute product endorsement.

* Corresponding author. Fax: +1 404 929 2686.

E-mail address: jstf2@cdc.gov (J.S. Fedan).

On the basis of these observations and the absence of information on the inhalation toxicology of popcorn flavorings, Hubbs et al. (2002) performed inhalation exposures of rats to butter flavoring. Respiratory epithelial cell necrosis in the upper and large lower airways was observed after exposure of the animals to constant or pulsed exposure (6 h) to flavoring (203–371 ppm diacetyl used as a marker). Subsequently, Hubbs et al. (2004) exposed rats to diacetyl alone and observed that most features of butter flavoring toxicity were recapitulated by this agent, although significant epithelial necrosis was limited to the nose and trachea.

Adams et al. (2000) observed that the respiratory epithelium may play a central role in the etiology of bronchiolitis obliterans. These investigators observed in a rat model that the presence of epithelium in isogeneic trachea grafts suppressed the development of obliteration. Earlier studies (Fedan et al., 2000) revealed profound alterations in tracheal epithelium histology after inhalation exposure of guinea pigs animals to O_3 , which accompanied increases in in vivo and in vitro airway reactivity. The morphological changes in epithelium induced by butter flavoring exposure in the studies by Hubbs et al. were more extensive and severe than those elicited by O_3 . Thus, the epithelium may be an especially sensitive initial target of the effects of popcorn flavoring in the lung, and it may be involved in the development of bronchiolitis obliterans induced by inhaled agents. Interestingly, ulceration of stomach glandular epithelium and hypertrophy of squamous stomach epithelium in rats were initiated by orally administered diacetyl (540 mg/kg for 90 days; Colley et al., 1969).

This study was performed to characterize the direct effects of diacetyl on two in vitro preparations of guinea pig trachea. Both allow an examination of the effects of the ketone in the absence of available inflammatory cells that might otherwise migrate into the airways during in vivo exposures. The isolated, perfused trachea preparation was used to assess possible changes in epithelial barrier function and airway reactivity to methacholine caused by diacetyl. An examination of the effects of diacetyl on the bioelectric properties of the epithelium was made using Ussing chambers. The effects of diacetyl itself, and the effect of diacetyl treatment on responses of the preparations to bronchoactive agents, were examined. The results indicate that the barrier function of the epithelium is degraded by the ketone, and this leads to hyperreactivity in vitro.

Materials and methods

Animals. These studies were conducted in facilities accredited fully by the Association for the Assessment and Accreditation of Laboratory Animal Care International and were approved by the institutional Animal Care and Use Committee. Male guinea pigs (589–750 g; Crl:HA) from Charles River (Wilmington, MA), monitored free of endogenous viral pathogens, parasites, and bacteria, were used in all experiments. The animals were acclimated before use and were housed in filtered ventilated cages on Alpha-Dri virgin cellulose chips and hardwood Beta-chips as bedding, provided HEPA-filtered air, Teklad 7006 diet and tap water ad libitum, under controlled light cycle (12 h light) and temperature (22–25 °C) conditions. The animals were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and sacrificed by thoracotomy and bleeding.

Mechanical responses of trachea: guinea pig isolated, perfused trachea preparation. This preparation permits separate delivery of agents to the

intraluminal (mucosal) or extraluminal (serosal) surfaces of the trachea and is useful for evaluating epithelial barrier function (Pavlovic et al., 1989; Munakata and Mitzner, 1991; Fedan and Frazer, 1992; Folkerts and Nijkamp, 1998) and the release of epithelium-derived relaxing factor (EpDRF; Munakata et al., 1990; McParland et al., 2000; Fedan et al., 2004). Briefly, a 4-cm tracheal segment was removed after anesthesia and mounted at in situ length on a holder for perfusion (34 ml/min) with modified Krebs–Henseleit solution (MKHS) while measuring, via catheters in the lumen attached to a differential pressure transducer, the inlet minus outlet pressure difference (ΔP , cm H_2O). The preparation was placed into a bath of MKHS (the extraluminal or serosal bath) and perfused at zero transmural pressure with re-circulating MKHS from a separate bath (intraluminal or mucosal bath). Increases and decreases in ΔP in response to challenge with contractile and relaxant agents, respectively, added to the extraluminal or intraluminal baths were recorded. The preparations were allowed a 1-h equilibration period before the experiment, during which the MKHS was changed at 15-min intervals.

Diacetyl concentration–response curves. Following the equilibration period, diacetyl was added in stepwise-increasing cumulative concentrations to the intraluminal bath of unstimulated tracheas or, in separate experiments, tracheas that had been contracted with extraluminal methacholine (MCh, 3×10^{-7} M), a muscarinic receptor agonist.

Responses of perfused trachea to MCh and terbutaline after diacetyl treatment. After the equilibration period, diacetyl (1, 3 or 10 mM) was added to the intraluminal MKHS. After a 4-h incubation with washes every 30 min with fresh MKHS containing diacetyl, the preparations were used in one of two ways: MCh was added cumulatively to the intraluminal or extraluminal baths to construct a concentration–response curve, or the preparations were contracted with extraluminal MCh (3×10^{-7} M) before terbutaline, a β_2 -adrenoceptor agonist, was added cumulatively to the extraluminal bath to generate a concentration–response curve. Separate tracheas were used as controls, and these were handled identically but in the absence of diacetyl.

Response to 120 mM KCl. After a 4-h incubation of tracheas in the presence or absence of intraluminal 3 mM diacetyl, the preparations were contracted with extraluminal MCh (3×10^{-7} M). At the plateau of the response, the tracheas were challenged with intraluminal 120 mM KCl. The purpose of this experiment was to test the ability of the epithelium to release EpDRF, and the basis for this experiment is as follows. EpDRF is released from epithelium in response to hyperosmolar challenge, and, in intact epithelia, all solutes used to raise intraluminal osmolarity, including KCl, are equipotent with regard to elicit a relaxation response (Fedan et al., 2004). If, however, the epithelium is damaged or unable to produce EpDRF, intraluminally applied KCl evokes a contraction response, as it does if it is applied to the extraluminal bath where it stimulates the smooth muscle directly or if it is added to an epithelial-denuded trachea (Fedan and Frazer, 1992).

Electrophysiological responses of tracheal epithelium to diacetyl: Ussing preparation. Tracheal segments at in situ length were mounted between the hemi-chambers of an Ussing apparatus and perfused continuously with MKHS. A pair of EKV (World Precision Instruments, Inc.) cartridge electrodes, each containing 4% agar in saline, were placed 3 mm from the orifice to detect transepithelial potential difference (V_t); a pair of EKC (World Precision Instruments, Inc.) cartridge electrodes, each containing 4% agar in saline, were placed within 2 cm of the orifice to deliver a calibrated current for determination of transepithelial resistance (R_t). Both EKV and EKC electrodes were connected to a voltage/current clamp amplifier (DVC 3000, World Precision Instruments, Inc). Electrode potential difference and fluid resistance were compensated before mounting the segments into the Ussing chamber. V_t was monitored under open-circuit conditions, and it usually reached stability within a 3-h equilibration period. Thereafter, V_t was recorded continuously while current pulses (5 μA square waves sustained for 5 s) were delivered every 50 s to yield a voltage response for calculation of R_t from Ohm's law. The data were logged on a strip-chart recorder (Gould, Inc.) and into data acquisition software (Acqknowledge™, Biopac, Inc.), from which the results were quantified. Both apical and basolateral baths were washed at 15-min intervals during the equilibration period.

At the end of the equilibration period, some preparations were challenged with MCh (3×10^{-7} M) added to the basolateral bath (to mimic conditions in the perfused trachea protocol), while others did not receive MCh. Diacetyl was added to the mucosal chamber in cumulative amounts to determine its effects on V_t and R_t .

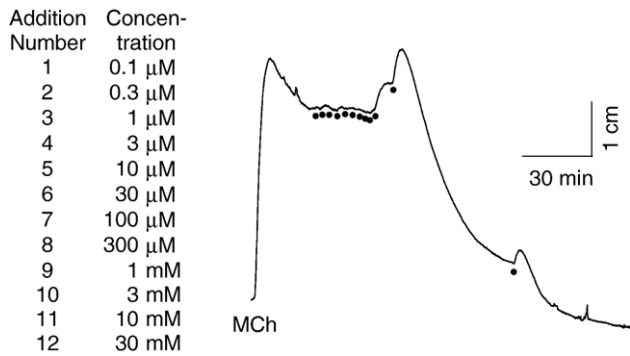


Fig. 1. Representative tracings showing the effects of intraluminally applied diacetyl in MCh(3×10^{-7} M)-contracted perfused tracheas. The dots under the tracings, from left to right, and the legend indicate the 12 cumulative additions that were made. The threshold for contraction was ca. 1–3 mM. Relaxation responses to 10 and 30 mM diacetyl were preceded by transient contractions. A summary of several experiments is shown in Fig. 2.

Solutions and reagents. MKHS contained 113 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl_2 , 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 25 mM NaHCO_3 , and 5.7 mM glucose, pH 7.4 at 37 °C, and was gassed with 95% O_2 and 5% CO_2 . All drugs, chemical, and agents were obtained from Sigma-Aldrich and dissolved and diluted in saline.

Analysis of results. The results are expressed as mean $\pm$ SE; n is the number of separate experiments. Geometric mean EC_{50} values were derived from least squares analysis of four-parameter logit curve fits. Statistical comparisons of EC_{50} values were done using normally distributed $-\log\text{EC}_{50}$ values. Results were analyzed for differences using Student's t test, Mann–Whitney rank sum test, or repeated measures ANOVA (RMANOVA), and the Student–Newman–Keuls post hoc method, as appropriate. $P < 0.05$ was considered significant.

Results

Diacetyl concentration–response relationships in perfused trachea

The direct effect of diacetyl was first examined. In uncontracted tracheas, diacetyl caused very weak contractions with the threshold at ca. 1 mM; above 3 mM, contractions were followed

by relaxation. When added in the presence of extraluminally added MCh (3×10^{-7} M; Figs. 1 and 2), contraction was elicited at ca. 3 mM diacetyl in most tracheas; 10 mM and 30 mM diacetyl also produced contractions, but these were transient and a net relaxation response predominated eventually.

Effect of diacetyl treatment on reactivity of perfused trachea to MCh

The effects of a 4-h perfusion with diacetyl on reactivity to intraluminal MCh (3×10^{-7} M) was studied using 1, 3, and 10 mM diacetyl. Fig. 3 and Table 1 show the results obtained with 1 and 3 mM diacetyl; 10 mM diacetyl inhibited completely responses to MCh. While 1 mM diacetyl was without a statistically significant effect, 3 mM diacetyl increased reactivity by ca. 10-fold; the maximum responses to MCh were not affected (Mann–Whitney rank sum test; $P > 0.05$).

In order to determine whether the increase in reactivity to intraluminal MCh caused by incubation with 3 mM diacetyl could be due to the ketone's effect on airway smooth muscle, concentration–response curves for extraluminally applied MCh were obtained. The results are shown in Fig. 4, where it can be seen that, when the results were plotted in terms of normalized responses, four responses at the higher MCh concentrations were potentiated. In fact, there was no effect of diacetyl on the EC_{50} value for MCh [$-\log\text{EC}_{50}$ (M) values were control, 7.26 ± 0.23 ; diacetyl, 6.85 ± 0.12 ; Student's nonpaired t test; $P > 0.05$] or the maximum responses [6.53 ± 1.04 and 4.74 ± 0.75 cm H_2O for control and diacetyl, respectively (Student's nonpaired t test; $P > 0.05$).

Effect of diacetyl treatment on reactivity of perfused trachea to terbutaline

We examined further the possibility that diacetyl might affect the smooth muscle by examining the effect of treatment with 3 mM diacetyl on reactivity to terbutaline. Diacetyl had no effect on sensitivity to terbutaline [$-\log\text{EC}_{50}$ (M) values were

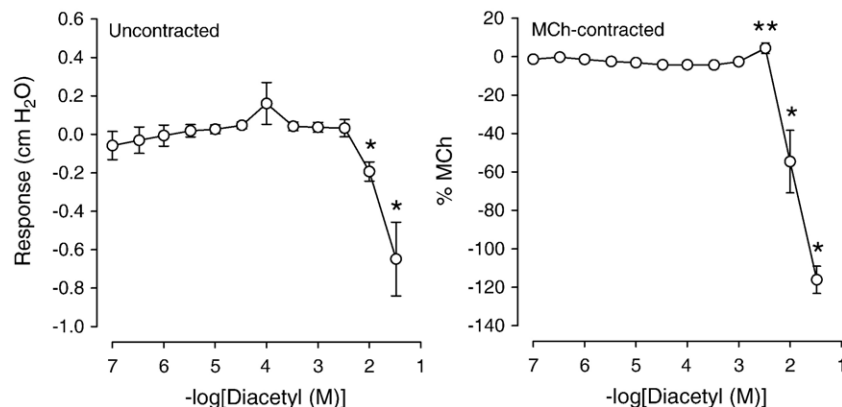


Fig. 2. Concentration–response relationships for the effects of intraluminally applied diacetyl in unstimulated (left panel, $n = 11$) and MCh-contracted perfused tracheas (right panel, $n = 10$). The responses in the left panel are quantified in terms of developed responses, ΔP , in cm H_2O . The responses in the right panel are expressed as a percentage of the MCh-induced tone at the time of diacetyl application. The responses shown for 10 and 30 mM are the net relaxation responses; transient contractions to these concentrations are not indicated in this plot. *Significant relaxation response; **Significant contractile response (RMANOVA with Student–Newman–Keuls post hoc method).

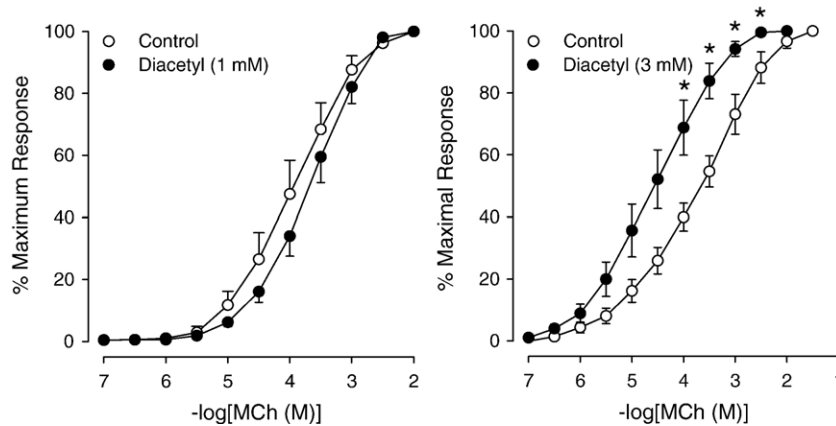


Fig. 3. Effect of a 4-h incubation with 1 mM diacetyl (left panel; $n = 6$) or 3 mM diacetyl (right panel; $n = 10$) in the intraluminal bath on reactivity to intraluminal MCh. 1 mM diacetyl had no effect on reactivity to MCh; however, 3 mM diacetyl increased sensitivity to MCh (Student's nonpaired t test; $P < 0.05$; see Table 1). The maximum response to MCh (cm H₂O) was not affected by either concentration of diacetyl. No responses to MCh were obtained after incubation with 10 mM diacetyl (not shown). *Significantly larger than control (Student's non-paired t test).

control, 7.44 ± 0.04 ; diacetyl, 7.41 ± 0.03 ; Student's nonpaired t test; $P > 0.05$] nor on the maximum response (Student's nonpaired t test; $P > 0.05$) ($n = 6$ for control and diacetyl-treated groups; results not shown).

Effect of diacetyl treatment on relaxant responses of perfused trachea to hyperosmolar solution

That the increase in reactivity to MCh after treatment with 3 mM diacetyl could have involved a decrease in the production of EpDRF was assessed by comparing the responses of control and diacetyl-treated tracheas to intraluminally applied KCl (120 mM). The relaxation response to KCl was not affected by diacetyl (Student's nonpaired t test; $P > 0.05$; $n =$ for control and diacetyl-treated groups; results not shown).

Effect of diacetyl treatment on V_t and R_t

The effects of diacetyl on V_t and R_t measured in Ussing preparations were evaluated in the absence and presence of basolaterally applied MCh (Fig. 5; Tables 2 and 3), inasmuch as MCh had been used to contract the smooth muscle in the perfused trachea experiments (see above). MCh elicited hyperpolarization (0.61 ± 0.22 mV). Under both conditions, concentrations of diacetyl (3 and 10 mM) which had evoked mechanical responses caused concentration-dependent depolar-

ization. MCh had no effect on responses expressed as the change in V_t . However, a significantly larger depolarizing response to 10 mM diacetyl occurred when the results were expressed in relation to the V_t measured before the addition of diacetyl ($\% \Delta V_t$ values were $-MCh$, 34.7 ± 20.2 and $+MCh$, 82.8 ± 3.9). MCh had no effect on R_t (before MCh, $117 \pm 33 \Omega \cdot \text{cm}^2$; after MCh, $127 \pm 23 \Omega \cdot \text{cm}^2$; Student's paired t test; $P > 0.05$). When the incubation with 10 mM diacetyl was allowed to continue for several hours, a progressive decrease in R_t occurred (Table 3). Fig. 5 shows examples in which the reductions in R_t were modest; this result was typical of 5 preparations in which MCh was absent and 4 preparations in which MCh was present. In one preparation (not shown), V_t decreased to near 0 mV during the application of 10 mM diacetyl, and R_t was all but abolished over the time period depicted in Fig. 5.

Discussion

The results of this study indicate that diacetyl alters the pharmacological and bioelectric characteristics of large airways.

Table 1
Effects of diacetyl on reactivity of perfused trachea to intraluminally applied MCh

	$-\log EC_{50}$ (M)	Maximum response (cm H ₂ O)
Control	3.93 ± 0.16	6.00 ± 1.64
1 mM Diacetyl	3.67 ± 0.16	4.46 ± 2.15
Control	3.47 ± 0.35	4.81 ± 1.05
3 mM Diacetyl	4.52 ± 0.25^a	3.88 ± 1.21

^a Significantly larger than control (Student's nonpaired t test). Maximum responses were not affected (Student's nonpaired t test; $P > 0.05$).

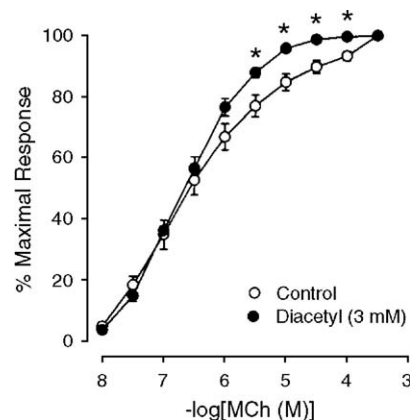


Fig. 4. Effect of a 4-h incubation with 3 mM diacetyl on reactivity to extraluminally applied MCh. *Significantly larger than control (Student's nonpaired t test). $n = 10$ for control and diacetyl-treated groups.

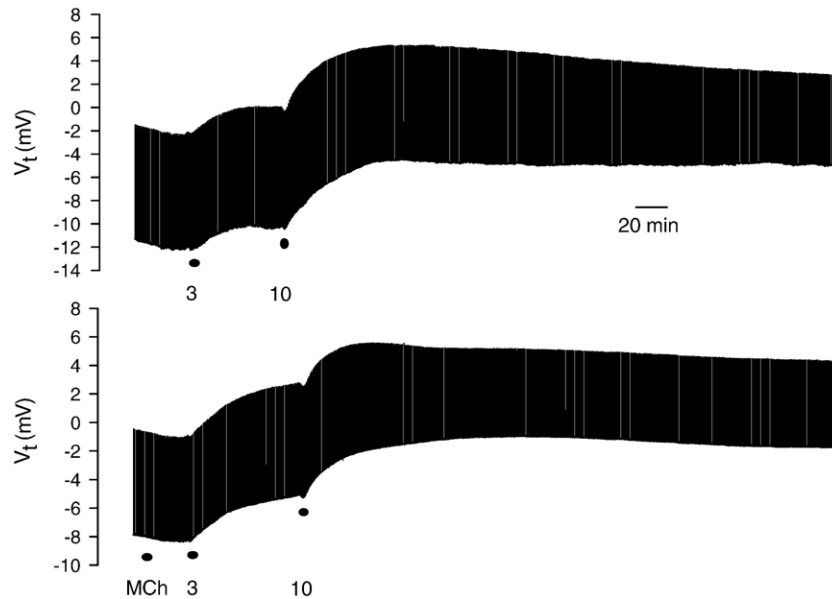


Fig. 5. Effects of 3 and 10 mM diacetyl in the absence (top panel) and presence (bottom panel) of 3×10^{-7} M MCh on V_t and R_t in tracheal segments mounted in Ussing chambers. The values at the lower limit of the band show V_b , and the V_t excursions at the upper limit, which are blended in this figure, are the result of the delivery of 5 μ A pulses that were delivered for the calculation of R_t . These tracings were obtained from parallel experiments performed at the same time on different tracheal segments.

The application of diacetyl to the apical surface of the guinea pig trachea evoked contraction and relaxation and caused damage of the epithelium which led to an increase in reactivity to mucosally—but not serosally—applied MCh. Diacetyl depolarized the epithelium and decreased R_t .

In unstimulated tracheas, intraluminally applied diacetyl led to very small contractions. The contractile responses were somewhat larger in tracheas that had first been contracted with MCh to induce tone. Under both experimental conditions, the contractions were followed by relaxations. It is possible that the relaxation phase of the response is a manifestation of toxicity because 10 mM diacetyl was observed to inhibit completely responses to extraluminally MCh applied. Contractile responses to diacetyl occurred as soon as the agent was administered; it is not known whether these responses were triggered by diacetyl per se or by substances from epithelial cells as they became damaged. These findings could suggest that a worker inhaling diacetyl might experience bronchoconstriction or bronchodila-

tion, depending on the tone in the airways and the amount of the ketone that is inhaled.

The increase in sensitivity to intraluminally applied but not extraluminally applied MCh after incubation with intraluminal diacetyl is clear evidence for an increase in reactivity that involved a change in epithelial permeability. It is somewhat remarkable that 1 mM diacetyl had no effect on reactivity to MCh, but that 3 mM diacetyl caused a ca. 10-fold increase. The finding that relaxation responses to extraluminal terbutaline were unaffected by diacetyl incubation is additional evidence for the notion that diacetyl's effects, at least at a concentration of 3 mM, were restricted to the epithelium.

In an earlier study, ozone exposure of guinea pigs led to an increase in reactivity to intraluminally applied MCh at a time

Table 2
 V_t responses of tracheal epithelium to apically applied diacetyl

Diacetyl concentration	MCh absent	MCh present ^b
	ΔV_t (mV) ^a	
3 mM (5)	0.95 ± 0.34	2.39 ± 0.83
10 mM (4)	3.64 ± 1.01	6.30 ± 0.99
	% ΔV_t	
3 mM (5)	14.9 ± 8.4	41.1 ± 20.2
10 mM (4)	34.7 ± 10.6	82.8 ± 3.9^c

^a Change in V_t (mV depolarization) from basal value in response to diacetyl.

^b MCh (3×10^{-7} M) was applied to the basolateral chamber.

^c Significantly larger than in the absence of MCh (Student's nonpaired *t* test).

n values are given in parentheses.

Table 3
Effect of apically applied diacetyl on R_t of tracheal epithelium

Condition	R_t ($\Omega \cdot \text{cm}^2$)	% ΔR_t ^a
MCh absent (5)		
Basal	117 ± 33	—
3 mM Diacetyl	123 ± 35	-5.0 ± 0.8
10 mM Diacetyl	79 ± 29^b	31.5 ± 14.6
MCh present (4)		
Basal ^c	127 ± 23	—
3 mM Diacetyl	140 ± 25	10.8 ± 2.2
10 mM Diacetyl	98 ± 33^d	28.0 ± 15.2

^a Percentage change in R_t from basal values in response to diacetyl.

^b Significantly different from basal and 3 mM (RMANOVA). MCh (3×10^{-7} M) was applied to the basolateral chamber.

^c Values used for basal were obtained in the presence of MCh, which were used for calculation of changes in response to diacetyl. MCh had no effect on R_t (see text); % ΔR_t after MCh addition was 0.4 ± 1.4 .

^d 10 mM vs. basal, $P < 0.077$ (RMANOVA). *n* values are given in parentheses.

when relaxation responses to intraluminal hyperosmolarity, i.e., EpDRF-mediated responses, were inhibited (Fedan et al., 2000). In that study, reactivity to MCh and hyperosmolar solution eventually returned to normal in the face of substantial morphological changes in the epithelium. In the present study, we observed that KCl-evoked, EpDRF-mediated responses were unaffected by 3 mM diacetyl, even though sensitivity to MCh was increased. Thus, diacetyl treatment had not interfered with the release and/or effects of EpDRF at a time when reactivity to MCh was increased. That is, the epithelial cells were not impaired to the degree that the direct, contractile effect of KCl on the smooth muscle overwhelmed the relaxant effect of released EpDRF.

Diacetyl depolarized the epithelium substantially and reduced R_t and, in one preparation, abolished R_t . The greater change in V_t than R_t suggests that diacetyl inhibited ion transport across the epithelium. The transport pathway(s) involved in these electrophysiological responses will need to be established in future experiments. The decrease in R_t may reflect a generalized permeability increase across the epithelium which may be of sufficient magnitude to contribute to the increased reactivity to intraluminal MCh.

Whereas workers are exposed by inhalation to popcorn flavoring vapor, our experiments on the in vitro effects of diacetyl have examined the effects of diacetyl in solution, which is an artificial exposure system. The concentrations of diacetyl that are achieved in airway surface liquid in workers have not been described, and it is difficult to extrapolate our current findings to the work site hazard. However, having established that diacetyl in solution alters airway epithelial function, our future experiments using delivery of diacetyl vapor in vitro will determine the vapor concentrations that produce an effect on epithelium and may allow us to compare these effects with those described in this report.

In conclusion, our results suggest that diacetyl exerts toxic effects on airway epithelium, which lead to airway hyperreactivity in vitro and degradation of its protective barrier function. The specific pathways involved in diacetyl's effects on epithelium require additional study. However, it is clear that the agent has untoward effects on epithelial cells under conditions in which inflammatory cells were not available from the blood. Mediators from resident inflammatory cells, which are limited in number in the airways from untreated animals, are not likely to have elicited the changes that were observed. The effects of diacetyl observed in this study may represent early functional changes in the epithelium in the airways of exposed workers.

References

- Adams, B.F., Brazelton, T., Berry, G.J., Morris, R.E., 2000. The role of respiratory epithelium in a rat model of obliterative airway disease. *Transplantation* 69, 661–664.
- Chan, A., Allen, R., 2004. Bronchiolitis obliterans: an update. *Curr. Opin. Pulm. Med.* 10, 133–141.
- Colley, J., Gaunt, I.F., Lansdown, A.B., Grasso, P., Gangolli, S.D., 1969. Acute and short-term toxicity of diacetyl in rats. *Food Cosmet. Toxicol.* 7, 571–580.
- Fedan, J.S., Frazer, D.G., 1992. Influence of epithelium on the reactivity of guinea-pig isolated, perfused trachea to bronchoactive drugs. *J. Pharmacol. Exp. Ther.* 262, 741–750.
- Fedan, J.S., Millecchia, L.L., Johnston, R.A., Rengasamy, A., Hubbs, A., Dey, R.D., Yuan, L.X., Watson, D., Goldsmith, W.T., Reynolds, J.S., Orsini, L., Dortch-Carnes, J., Cutler, D., Frazer, D.G., 2000. Effect of ozone treatment on airway reactivity and epithelium-derived relaxing factor in guinea pigs. *J. Pharmacol. Exp. Ther.* 293, 724–734.
- Fedan, J.S., Dowdy, J.A., Johnston, R.A., Van Scott, M.R., 2004. Hyperosmolar solution effects in guinea pig airways: I. Mechanical responses to relative changes in osmolarity. *J. Pharmacol. Exp. Ther.* 308, 10–18.
- Folkerts, G., Nijkamp, F.P., 1998. Airway epithelium: more than just a barrier! *Trends Pharmacol. Sci.* 19, 334–341.
- 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. Pharmacol.* 185, 128–135.
- Hubbs, A., Battelli, L.A., Mercer, R.R., Kashon, M.L., Friend, S., Schwegler-Berry, D., Goldsmith, W.T., 2004. Inhalation toxicity of the flavoring agent, diacetyl (2,3-butanedione), in the upper respiratory tract of rats. *Toxicol. Sci.* 78, 438.
- 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.
- Kullman, G., Boylstein, R., Jones, W., Piacitelli, C., Pendergrass, S., Kreiss, K., 2005. Characterization of respiratory exposures at a microwave popcorn plant with cases of bronchiolitis obliterans. *J. Occup. Environ. Hyg.* 2, 169–178.
- Laohaburanakit, P., Chan, A., Allen, R.P., 2003. Bronchiolitis obliterans. *Clin. Rev. Allergy Immunol.* 25, 259–274.
- McParland, B.E., Johnson, P.R., Armour, C.L., Black, J.L., 2000. An epithelial-derived factor inhibits contraction in canine perfused bronchial segments. *Eur. J. Pharmacol.* 402, 151–159.
- Munakata, M., Mitzner, W., 1991. The protective role of the airway epithelium. In: Farmer, S.G., Hay, D.W.P. (Eds.), *The Airway Epithelium*. Marcel Dekker, Inc., New York, pp. 545–564.
- Munakata, M., Masaki, Y., Sakuma, I., Ukita, H., Otsuka, Y., Homma, Y., Kawakami, Y., 1990. Pharmacological differentiation of epithelium-derived relaxing factor from nitric oxide. *J. Appl. Physiol.* 69, 665–670.
- Pavlovic, D., Fournier, M., Aubier, M., Pariente, R., 1989. Epithelial vs. serosal stimulation of tracheal muscle: role of epithelium. *J. Appl. Physiol.* 67, 2522–2526.
- Schachter, E.N., 2002. Popcorn worker's lung. *N. Engl. J. Med.* 347, 360–361.
- Scott, A.I., Sharples, L.D., Stewart, S., 2005. Bronchiolitis obliterans syndrome: risk factors and therapeutic strategies. *Drugs* 65, 761–771.
- Shaw, R.J., Djukanovic, R., Tashkin, D.P., Millar, A.B., du Bois, R.M., Orr, P.A., 2002. The role of small airways in lung disease. *Respir. Med.* 96, 67–80.