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Accumulation of Ubiquitin and Sequestosome-1 Implicate Protein Damage in Diacetyl-Induced Cytotoxicity

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Inhaled diacetyl vapors are associated with flavorings-related lung disease, a potentially fatal airway disease. The reactive α-dicarbonyl group in diacetyl causes protein damage *in vitro*. Dicarbonyl/L-xylulose reductase (DCXR) metabolizes diacetyl into acetoin, which lacks this α-dicarbonyl group. To investigate the hypothesis that flavorings-related lung disease is caused by *in vivo* protein damage, we correlated diacetyl-induced airway damage in mice with immunofluorescence for markers of protein turnover and autophagy. Western immunoblots identified shifts in ubiquitin pools. Diacetyl inhalation caused dose-dependent increases in bronchial epithelial cells with puncta of both total ubiquitin and K63-ubiquitin, central mediators of protein turnover. This response was greater in *Dcxr*-knockout mice than in wild-type controls inhaling 200 ppm diacetyl, further implicating the α-dicarbonyl group in protein damage. Western immunoblots demonstrated decreased free ubiquitin in airway-enriched fractions. Transmission electron microscopy and colocalization of ubiquitin-positive puncta with lysosomal-associated membrane proteins 1 and 2 and with the multifunctional scaffolding protein sequestosome-1 (SQSTM1/p62) confirmed autophagy. Surprisingly, immunoreactive SQSTM1 also accumulated in the olfactory bulb of the brain. Olfactory bulb SQSTM1 often congregated in activated microglial cells that also contained olfactory marker protein, indicating neuronophagia within the olfactory bulb. This suggests the possibility that SQSTM1 or damaged proteins may be transported from the nose to the brain. Together, these findings strongly implicate widespread protein damage in the etiology of flavorings-related lung disease. *(Am J Pathol 2016, 186: 2887–2908; http://dx.doi.org/10.1016/j.ajpath.2016.07.018)*

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In 2000, a group of eight workers at a microwave popcorn manufacturing plant were diagnosed with clinical bronchiolitis obliterans, with severe fixed airways obstruction.[1] Workers at the plant were found subsequently to have increased rates of lung disease and airways obstruction relative to the general population. The altered lung function of those workers correlated with exposure to diacetyl (2,3-butanedione), a flavoring present in natural butter and a common component of butter flavoring.[1] Studies in rats and mice demonstrated remarkable airway epithelial cytotoxicity after diacetyl inhalation.[2–4] In workers, high-resolution computed tomography scans demonstrated bronchial wall thickening in eight popcorn manufacturing workers, and two workers had biopsy findings consistent with constrictive bronchiolitis.[5] Additional cases of fixed airways obstruction were subsequently identified in workers exposed to diacetyl vapors in other workplaces; those workplaces involved microwave popcorn, coffee, chemical, and flavorings production.[6–9] Recently, workers in flavoring production were found to have an increased prevalence of restrictive lung disease, suggesting that the full spectrum of pulmonary function abnormalities associated with inhaling flavoring vapors may include restrictive as well as obstructive lung disease.[10] The occupational disease in workers is known as popcorn workers' lung or flavorings-related lung disease. Additional high-concentration diacetyl inhalation exposures occur in traditional cigarette smokers and e-cigarette vapers.[11,12]

Diacetyl is a four-carbon compound with adjacent carbonyl carbons, putting it into a class of compounds known as α-dicarbonyl compounds. The adjacent carbonyl groups tend to increase the chemical reactivity of α-dicarbonyl compounds.[13,14] The importance of these adjacent carbonyl groups was recently underlined by the discovery that another α-dicarbonyl butter flavoring, 2,3-pentanedione, caused airway epithelial injury and airway fibrosis in rats, which was similar to diacetyl-induced airway injury.[15,16] Protein damage, particularly at arginine residues, can be caused by chemical interactions between proteins and diacetyl or the structurally related 3-carbon α-dicarbonyl compound, methylglyoxal.[17,18] Although most studies of α-dicarbonyl compounds are conducted *in vitro,* a recent study demonstrated arginine adducts in hemoglobin and albumin of rats and mice after intratracheal instillation or oropharyngeal aspiration of [^{14}C]diacetyl *in vivo*.[19] This suggests that protein damage occurs *in vivo* and could play a major role in the development of flavorings-related lung disease. However, neither protein damage nor biological responses to excessive protein damage have been identified in the airway epithelium after diacetyl inhalation.

Damaged proteins can fail to form the desired three-dimensional shape, resulting in unfolded or misfolded proteins. Unfolded and misfolded proteins are often cytotoxic.[20,21] Misfolded proteins tend to form aggregates. Aggregated proteins are extraordinarily toxic to cells, generally inaccessible to the proteasome, and sometimes form recognizable ubiquitin-containing inclusions or puncta within cells.[22] Biological adaptation to misfolded proteins commonly involves the ubiquitin system.[20] Ubiquitin is a 76–amino acid protein that conjugates with misfolded proteins, enabling their degradation into small peptides within the proteasome.[20,23–25] Ubiquitin conjugation most frequently occurs at one of the seven lysine residues present in ubiquitin. These seven sites are K6, K11, K27, K29, K33, K48, and K63. Conjugation at the different lysine residues of ubiquitin can have different consequences for proteins, a situation that is best described for K48- and K63-linked ubiquitination. Ubiquitin-mediated protein degradation in the proteasome principally involves K48-linked polyubiquitin chains and is generally regarded as a process that protects the cell from damaged proteins and preserves amino acids. By contrast, proteins linked by K63 ubiqutination generally do not undergo proteasomal degradation; in fact, K63-linked ubiquitination is enhanced by inhibition of the proteasome.[26] K63-linked ubiquitination can affect cellular signaling, DNA repair, endosomal trafficking, and clearance of aggregated proteins through autophagy.[27–29] Ubiquitin-mediated autophagy in the lysosome is particularly useful in eliminating aggregated proteins.[21,30] However, excessive autophagy of proteins can lead to cell death and potentially causes ubiquitin and amino acid depletion. This is important because ubiquitin plays an essential role in both protein quality control and regulating proteins important in signaling cascades.[20,23–25,31,32] In addition, several important human diseases involve intracellular accumulations of ubiquitin and protein aggregates.[33–35] Thus, it is clear that the appearance of increased numbers of ubiquitin-positive puncta in conjunction with autophagy is a well-described response to altered protein homeostasis. However, it is not known if diacetyl inhalation alters these processes *in vivo* in the intrapulmonary airway epithelial cells, which are the target tissue in flavorings-related lung disease.

Diacetyl can be metabolized by the enzyme, dicarbonyl/L-xylulose reductase (DCXR).[36] The product of DCXR-mediated diacetyl metabolism is acetoin, which contains an α-hydroxyketone in place of the reactive α-dicarbonyl group. In an acute inhalation toxicity mixed exposure study using ratios similar to those seen in workplaces, acetoin did not significantly alter diacetyl-induced changes in airway reactivity.[37] This provides additional support for the role of the reactive α-dicarbonyl group in diacetyl-induced toxicity and suggests the possibility that DCXR may provide a degree of protection from diacetyl-induced airway injury by removing the α-dicarbonyl group implicated in causing protein damage.

In this study, we investigated the hypothesis that flavorings-related lung disease is caused by *in vivo* protein damage. Using immunofluorescence microscopy, morphometry, confocal microscopy, and transmission electron microscopy, we provide strong evidence that diacetyl disrupts protein homeostasis in the lung, and this is localized to the airway epithelium, the target tissue in flavorings-related lung