The Flying Monkeys of Ozone: Oxysterols Inactivate NLRP2 in Airway Epithelial Cells

The proinflammatory effects of the toxic air pollutant ozone (O_3) have been well established, yet the direct mechanisms by which O_3 inhalation impacts airway epithelial cells remain unclear. In this issue of the *Journal*, Perryman and colleagues (pp. 500–512) report NLRP2 as a highly expressed regulator of O_3-induced inflammatory responses in airway epithelial cells (1). NLR (Nucleotide-binding Oligomerization Domain, NOD-Like Receptor) proteins were originally identified as the main part of inflammasomes (caspase-activating complexes) together with the adaptor ASC (Apoptosis-associated Speck-like protein Containing a CARD) and pro–caspase-1 (2). NLRs belong to the innate immune pattern recognition receptor family responsible for the recognition of pathogen-associated or tissue-injury–associated molecular patterns. The canonical inflammasome activation process culminates in the production of active caspase-1 that cleaves pro–IL-1β into mature IL-1β, making inflammasomes crucial mediators of acute inflammation. As such, their formation and activation and the expression of NLRs are under complex genetic, epigenetic, transcriptional, and posttranslational control (3, 4). To date, several types of inflammasomes (NLRPs, AIM2, IFI [IFN-γ inducible factor] 16, and RIG-I) have been characterized. Although the NLR structure (containing an N-terminal pyrin domain, central NACHT [NAIP (NLP family apoptosis inhibitor protein), CIITA (C2TA or MHC class II transcription activator), HET-E (incompatibility locus protein from *Podospora anserina*) and TEP1 (TP1 or telomerase-associated protein)] domain, and C-terminal leucine rich repeat [LRR]) is well conserved, the NLR protein family shows a wide range of tissue-specific expression patterns and biological functions (3–6). NLRs interact with pathogen- and/or danger-associated molecules through unique mechanisms, and this interaction does not necessarily lead to inflammasome complex formation. For example, NLRP3—the most studied inflammasome-forming NLR family member (predominant in myeloid-derived cells)—is highly proinflammatory (3), whereas NLRP4 and NLRP7 are antiinflammatory molecules, and NLRP2, NLRP5, and NLRP7 are also involved in nonimmune pathways such as embryonic development (6).

The authors examined the expression of NLRP2 and four additional NLRP family members (NLRP1, NLRP3, NLRP7, and NLRP12) in different human respiratory epithelial cell culture models including primary differentiated human bronchial epithelial cells and human airway epithelial cell lines (16HBE, BEAS2B, and A549). These cells showed significantly greater NLRP2 mRNA and protein expression than the other NLR family members measured. Furthermore, THP-1 cells, a human blood monocyte cell line, and K562 cells, an erythroleukemia cell line, expressed high levels of NLRP3 and NLRP1, respectively, but not NLRP2, confirming the tissue specificity in expression of these NLR family members.

NLRP2, a poorly understood member of the NLR family, is implicated in diverse biological functions ranging from fertility in oocytes (7) to pain in dorsal ganglia (8) to inflammasome formation and IL-1β release in monocytes and astrocytes. NLRP2 was also reported as an inhibitor of NF-κB and a regulator of caspase-1 activity in placenta, liver, astrocytes, and immune cell types (summarized in the article by Perryman and colleagues [1]). To date, the function of NLRP2 in lung epithelial cells has not been well characterized, and its role in O_3-exposure–induced inflammation is not known.

Inhalation of O_3 activates inflammasome and NF-κB, cytokine and mediator release, and neutrophil influx into the airways (9–11). O_3 also reacts with unsaturated bonds found in phospholipids such as cholesterol (12), transforming it to oxysterols. Oxysterols are electrophiles that form covalent linkages preferentially with lysine residues, consequently modifying protein function. Lipid oxidation products by themselves may reproduce the O_3-induced activation of NF-κB and proinflammatory signaling (reviewed by Perryman and colleagues [1]). Similarly to their previous studies (13), Perryman and colleagues used airway epithelial cells treated with alkynyl-modified oxysterols. They then reacted the cell lysates with an azido biotin reagent under click cycloaddition conditions causing protein biotinylation on covalent bonds with alkynyl-tagged Secosterol-A (a-SecoA). By shotgun proteomics, 135 novel SecoA–protein adducts were identified in the treated cells (1). Among them was NLRP2 forming a SecoA–protein adduct at lysine (K1019) in the terminal LRR, a known regulatory region for NLR proteins. To verify that the same SecoA adducts are also formed from endogenous cholesterol, the authors also used alkynyl-tagged cholesterol treatment followed by O_3 exposure (Figure 1).

The most abundant proteins (e.g., chaperones, tubulin, actin, laminin) could be adducted by oxysterols in a cell-type–dependent, time-dependent, and dose-dependent fashion, ultimately leading to compromised membrane integrity, endoplasmic reticulum stress, apoptosis, or NF-κB and unfolded protein response pathways (1, 14) (and reviewed by authors of Reference 1). Owing to its abundance in airway epithelial cells and known regulatory functions in other cell types, NLRP2 may be an important early target for oxysterol adduction. Interestingly, of the 66 lysine residues in NLRP2, only K1019 in the terminal LRR was modified. NLRP3—which is highly expressed in the monocytic THP-1 cells studied here—was not adducted by SecoA, suggesting selectivity and specificity of epithelial cell oxysterol adduct formation. The authors aligned the LRR regions for NLRP2 and NLRP3 and reasoned that the hydrophobic nature of LRRs combined with the specific local NLRP2 primary sequence (containing an enrichment of acidic amino acids flanking either side of the SecoA-modified lysine residue within the conserved motif [LxxLxLxxDx] [1, 13, 15]) attracts SecoA to the horseshoe-shaped

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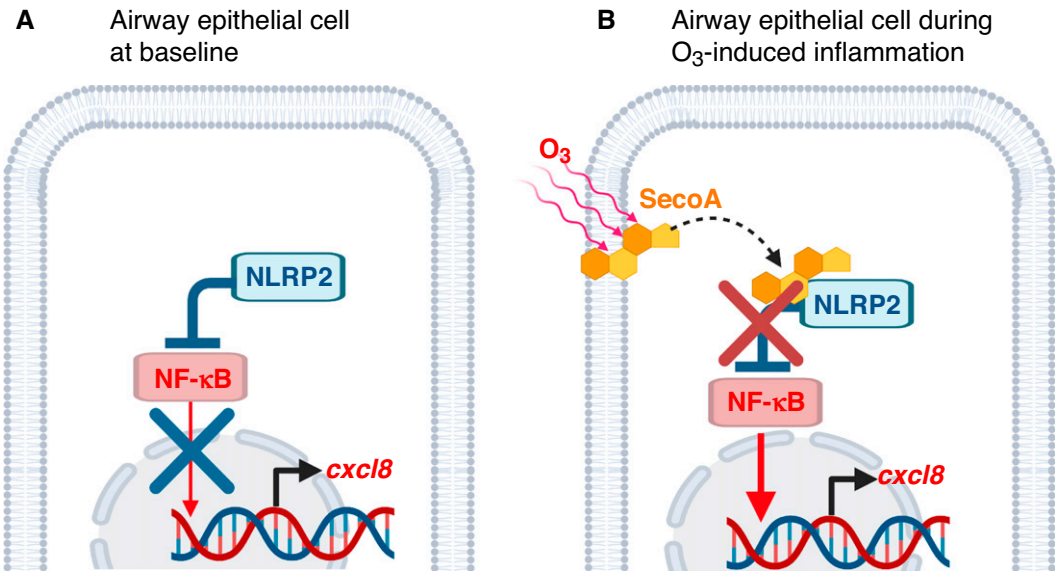

Figure 1. Oxysterols impair the protective effect of NLRP2 on NF-κB activation in airway epithelial cells. (*A*) NLRP2 is abundantly expressed and inhibits NF-κB, thus preventing inflammatory gene (such as *cxcl8*) transcription at baseline in airway epithelial cells. (*B*) O_3 reacts with unsaturated bonds of cholesterol, transforming it into oxysterols (such as Secosterol-A [SecoA]). SecoA binds to NLRP2 and forms an adduct at lysine (K1019), thus inactivating NF-κB suppression and promoting inflammatory gene (*cxcl8*) transcription. NLRP2 = NLR family pyrin domain containing 2; O_3 = ozone.

LRR region and allows the electrophilic attack of the single lysine residue in this prominent region.

O_3-induced caspase-1 and inflammasome activation were previously reported (16, 17) in macrophages and other immune cells that likely contribute to inflammation via the NLRP3 inflammasome complex (18). The functional significance of NLRP2 in airway epithelial cells was investigated by CRISPR-Cas9 knockout and shRNA knockdowns that increased the expression of CXCL1, CXCL2, and CXCL8/IL-8 in response to O_3, confirming a noncanonical inhibition of NF-κB signaling. There was no colocalization of NLRP2 and ASC or formation of ASC–NLRP2 complex after O_3 exposure on the basis of immunohistochemistry or IP assessments, suggesting that inflammasome or caspase-1 activation is not involved in the action of this NLR member in the O_3 response. MAPK signaling, however, was implicated before (19) and should be explored in future studies.

To study whether adduction by O_3-derived oxysterols at K1019 was responsible for inactivating NLRP2's ability to inhibit proinflammatory signaling, using site-directed mutagenesis, the authors reconstituted NLRP2-deficient epithelial cells with a K1019R mutant that significantly (albeit not fully) blocked SecoA adduction. Similarly, replacement of NLRP2 with the wild-type gene attenuated O_3-induced proinflammatory responses. But the K1019R mutation resulted in an intermediary phenotype of O_3-induced IL-8 secretion. Such partial blockade suggests the existence of additional sites of SecoA modification of NLRP2 that were not covered by their shotgun proteomic analysis, acknowledged as a limitation of this study. The authors speculate that, in addition to K1019 in the LRR of NLRP2, the pyrin and NACHT domains (both shown to inhibit NF-κB) could also play a role (20). Thus, oxysterol adduction at K1019 mediates (at least partially) the effects of O_3 and inactivates the antiinflammatory function of NLRP2 in epithelial cells (Figure 1). The significance of these *in vitro* findings would need *in vivo* corroboration. This is, however, a challenge given that human and murine NLRP2 appear to have marked functional discrepancies (21).

In summary, this interesting study suggests that, in human airway epithelial cells, NLRP2 is highly expressed and plays a noncanonical antiinflammatory function. This homeostatic role is then impaired by a specific O_3-induced inactivation of a regulatory sight in the molecule through oxysterol adduct formation. Aside from the limitations, this work paves the way for further investigations into the complex roles of cholesterol-derived oxidative products and NLRPs in inflammatory regulation of the airways. ■

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