The Journal of Immunology

Aeroallergens Induce Reactive Oxygen Species Production and DNA Damage and Dampen Antioxidant Responses in Bronchial Epithelial Cells

Tze Khee Chan, W. S. Daniel Tan, Hong Yong Peh and W. S. Fred Wong

J Immunol 2017; 199:39-47; Prepublished online 19 May 2017;
doi: 10.4049/jimmunol.1600657
http://www.jimmunol.org/content/199/1/39

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Aeroallergens Induce Reactive Oxygen Species Production and DNA Damage and Dampen Antioxidant Responses in Bronchial Epithelial Cells

Tze Khee Chan,*,†,‡,[1] W. S. Daniel Tan,*,†,[1] Hong Yong Peh,*,† and W. S. Fred Wong*,†,§

Exposure to environmental allergens is a major risk factor for asthma development. Allergens possess proteolytic activity that is capable of disrupting the airway epithelium. Although there is increasing evidence pointing to asthma as an epithelial disease, the underlying mechanism that drives asthma has not been fully elucidated. In this study, we investigated the direct DNA damage potential of aeroallergens on human bronchial epithelial cells and elucidated the mechanisms mediating the damage. Human bronchial epithelial cells, BEAS-2B, directly exposed to house dust mites (HDM) resulted in enhanced DNA damage, as measured by the CometChip and the staining of DNA double-strand break marker, γH2AX. HDM stimulated cellular reactive oxygen species production, increased mitochondrial oxidative stress, and promoted nitrosative stress. Notably, expression of nuclear factor erythroid 2–related factor 2–dependent antioxidant genes was reduced immediately after HDM exposure, suggesting that HDM altered antioxidant responses. HDM exposure also reduced cell proliferation and induced cell death. Importantly, HDM-induced DNA damage can be prevented by the antioxidants glutathione and catalase, suggesting that HDM-induced reactive oxygen and nitrogen species can be neutralized by antioxidants. Mechanistic studies revealed that HDM-induced cellular injury is NADPH oxidase (NOX)-dependent, and apocynin, a NOX inhibitor, protected cells from double-strand breaks induced by HDM. Our results show that direct exposure of bronchial epithelial cells to HDM leads to the production of reactive oxygen and nitrogen species that damage DNA and induce cytotoxicity. Antioxidants and NOX inhibitors can prevent HDM-induced DNA damage, revealing a novel role for antioxidants and NOX inhibitors in mitigating allergic airway disease. ***The Journal of Immunology*****, 2017, 199: 39–47.**

Allergic disease continues to increase in prevalence, affecting ∼30–40% of the world's population (1). It is a maladaptive immune response directed against noninfectious environmental substances known as allergens (2, 3). Allergic diseases caused by airborne allergens include rhinoconjunctivitis, eosinophilic bronchitis, and allergic asthma. Allergic asthma is an inflammatory disorder of the airways toward airborne allergens such as house dust mites (HDM), animal dander, plant pollen, and fungal spores (4). These aeroallergens may have deleterious impacts on the bronchial epithelium, contributing to asthma development. The process of allergic sensitization of the airway cells to aeroallergens is well investigated, but study of the damaging impacts of allergens on lung airway epithelial cells is lacking.

Airway epithelial cells constitute the first line of defense against environmental stimuli and pathogens such as viruses, pollutants, and allergens (5). Particularly, the bronchial epithelium is a key regulator of airway allergen sensitization and remodeling. Allergens may damage epithelial integrity through their protease activity and cytotoxic potential (4). Disruption of the epithelial cell barrier and intercellular adhesion enables easier access of pathogens into the airways, which could be one of the contributory factors in the development of asthma (6). In addition, allergen-mediated activation of pattern recognition receptors and protease-activated receptors, and induction of reactive oxygen species (ROS) are critical steps to the initiation of inflammatory responses (4). One of the key producers of ROS is NADPH oxidase (NOX), a transmembrane protein responsible for the transportation of electrons across the membrane, which at the same time reduces oxygen to superoxide ($\cdot O_2^-$) (7). NOX-derived ROS plays a critical role in host defenses and cellular signaling, however, excessive production of ROS leads to cellular stress (7). In this study, we will evaluate the contribution of NOX-derived ROS in HDM-induced epithelial cell injury.

Oxidants are known to increase epithelial permeability by damaging tight junctions. Overproduction of reactive oxygen and nitrogen species (RONS) is harmful to cells, as they induce oxidative and nitrosative damage to cellular macromolecules such as the nucleic acid. RONS react with DNA to form a broad range of

*Department of Pharmacology, Yong Loo Lin School of Medicine, National University Health System, Singapore 117600, Singapore; †Immunology Program, Life Science Institute, National University of Singapore, Singapore 117456, Singapore; ‡Infectious Diseases Interdisciplinary Research Group, Singapore-MIT Alliance for Research and Technology, Campus for Research Excellence and Technological Enterprise, Singapore 138602, Singapore; and §Molecular Mechanisms of Inflammatory Diseases Interdisciplinary Research Group, Singapore-HUJ Alliance for Research and Enterprise, Campus for Research Excellence and Technological Enterprise, Singapore 138602, Singapore

[1]T.K.C. and W.S.D.T. contributed equally to this work.

ORCID: 0000-0002-5840-5698 (W.S.D.T.).

Received for publication April 14, 2016. Accepted for publication April 21, 2017.

This work was supported by the National Medical Research Council (Grant NMRC/CBRG/0027/2012) and the National University Health System (Seed Grant R-184-000-238-112). This study was also supported by the National Research Foundation Singapore through the Singapore-MIT Alliance for Research and Technology program, as well as Campus for Research Excellence and Technological Enterprise Grant R-184-000-269-592.

Address correspondence and reprint requests to Prof. W. S. Fred Wong, Department of Pharmacology, Yong Loo Lin School of Medicine, National University Health System, Block MD3, 16 Medical Drive, Level 4, #04-01, Singapore 117600, Singapore. E-mail address: phcwongf@nus.edu.sg

The online version of this article contains supplemental material.

Abbreviations used in this article: AIF, apoptosis-inducing factor; ASP, *Aspergillus fumigatus*; CAE, cockroach allergen extract; DDR, DNA damage response; DSB, double-strand break; HDM, house dust mite; iNOS, inducible NOS; NOS, NO synthase; NOX, NADPH oxidase; Nrf2, nuclear factor erythroid 2–related factor 2; PI, propidium iodide; RONS, reactive oxygen and nitrogen species; ROS, reactive oxygen species; RWE, ragweed pollen extract; SOD, superoxide dismutase; XO, xanthine oxidase.

DNA lesions including base damage, single-strand breaks, and double-strand breaks (DSBs). DNA damage in cells triggers the activation of the DNA damage response (DDR). DDR is a coordinated series of cellular pathways that detect, signal, and repair DNA lesions to prevent the generation of potentially deleterious mutations (8). DDR includes the activation of cell-cycle checkpoint mechanisms to arrest the cell cycle for DNA damage repair or the activation of cell death pathway, if the damage is too severe to be repaired (8).

To the best of our knowledge, this is the first demonstration that the HDM aeroallergen directly induces DNA damage in human bronchial epithelial cells, through the production of genotoxic RONS, probably via enhanced NOX2 and NOX4 gene expression, with associated increases in cellular nitrosative and mitochondrial oxidative stress. HDM exposure alters antioxidant responses in epithelial cells, induces apoptosis-inducing factor (AIF) nuclear translocation, triggers apoptosis, and reduces cell proliferation. This highlights the cytotoxic potential of HDM on the airway epithelium, which could be rescued by the NOX inhibitor apocynin, and antioxidants such as glutathione and catalase in a concentration-dependent manner, giving direct evidence that NOX inhibitors and antioxidants protect airway cells from the damaging effects of aeroallergens. Our findings have direct implications in asthma disease etiology. A better understanding of the function of epithelial cells in maintaining airway integrity and how its dysfunction contributes to asthma enables a deeper understanding of the mechanisms by which asthma is initiated, and provides a framework to identify new therapeutic strategies to mitigate the disease.

Materials and Methods

Reagents

HDM extract, cockroach allergen extract (CAE), *Aspergillus fumigatus* (ASP), and ragweed pollen extract (RWE) were obtained from Greer Laboratories (Greerlab, Lenoir, NC). The endotoxin content in 1, 10 and 100 μg HDM extracts are 0.005, 0.05 and 0.5 μg/ml respectively.

Cell culture and treatment

BEAS-2B cells obtained from American Type Culture Collection (Rockville, MD) were grown in bronchial epithelium growth medium (BEGM BulletKit; Lonza, Basel, Switzerland) in flasks precoated with 30 μg/ml collagen (Sigma-Aldrich, St. Louis, MO) and 10 μg/ml fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were plated on six-well plates and treated with HDM protein (1, 10, 100 μg) dissolved in culture medium for 6 or 20 h. In antioxidant treatment, cells were exposed to 100 μg of HDM in the presence of glutathione (0.1, 1, 10 mM) (Sigma-Aldrich) or catalase (2, 20, 200 U/ml) (Sigma-Aldrich) for 6 h. In oxidase inhibitor studies, cells were pretreated with the NOX inhibitor apocynin (100 μM) (Sigma-Aldrich) or the xanthine oxidase (XO) inhibitor oxypurinol (100 μM) (Sigma-Aldrich) for 1 h before coincubating with 100 μg of HDM for 6 h. Untreated control was replenished with fresh culture medium.

Comet assay using CometChip

CometChip is a highly sensitive and higher-throughput version of the comet assay that leverages micropatterning of microwells to create arrayed comets (9–11). Arrayed microwells were prepared as described by Wood et al. (10). BEAS-2B cells were loaded into the microwells and exposed to different treatment conditions. Cells were incubated with HDM with or without the presence of antioxidants glutathione or catalase (Sigma-Aldrich), or TLR-4 antagonist (#tlrl-prslps, LPS from *Rhodobacter sphaeroides*; InvivoGen, San Diego, CA) for 6 h. To mimic selective activation of TLR-4, cells were treated with TLR-4 agonist (#tlrl-3pelps, LPS from *Escherichia coli O111: B4*; InvivoGen) for 6 h without HDM. Control cells were exposed to hydrogen peroxide (H_2O_2) (100 μM) with or without the presence of antioxidants glutathione or catalase. After treatment, alkaline CometChip assay was performed using protocol as described by Weingeist et al. (11).

Immunofluorescence staining

Cells were grown on four-well culture glass chamber slides and exposed to different treatment conditions. Cells were then fixed in ice-cold methanol, immunoblocked with 5% BSA, and probed with primary Abs targeting γH2AX or AIF (Cell Signaling Technology, Danvers, MA), followed by secondary Ab and DAPI staining. Bleomycin (Sigma-Aldrich)-treated cells (0.1 U/ml) were used as a positive control in γH2AX staining. H_2O_2 (100 μM)-treated cells were used as a positive control in CellROX staining. For CellROX and MitoTracker Red CM-H_2XRos (Thermo Fisher Scientific, San Jose, CA) staining, slides were stained according to the manufacturer's instructions. All slides were mounted in SlowFade Gold antifade reagent (Thermo Fisher Scientific). Images were captured using the Zeiss AxioImager Z1 microscope (Zeiss, Toronto, ON, Canada) and quantitated using Imaris 5.7 software (Bitplane AG, Zurich, Switzerland).

Quantitative real-time PCR analysis

Total RNA was extracted from BEAS-2B cells with RNAzol RT (Molecular Research Center, Cincinnati, OH). cDNA was synthesized using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD), and subjected to quantitative PCR. Briefly, template cDNA was mixed with SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) and was quantified using a sequence detector (ABI 7500 Cycler; Applied Biosystems, Foster City). The mRNA expression of antioxidant genes listed in Supplemental Table I was normalized to the level of the housekeeping gene GAPDH.

Immunoblotting

BEAS-2B cell nuclear protein extraction was performed using a nuclear extraction kit (Active Motif, Carlsbad, CA). Nuclear protein extracts were separated by 10% SDS-PAGE and immunoblots were probed with anti–nuclear factor erythroid 2–related factor 2 (Nrf2) Ab or anti-Lamin A/C Ab (Cell Signaling Technology). Cells treated with Nrf2 activator sulforaphane (10 μM) (Sigma-Aldrich) for 4 h were used as a positive control. To quantify the level of γH2AX in HDM extracts alone, HDM extract lysates (1, 10 and 100 μg) were separated by SDS-PAGE and probed for γH2AX (Cell Signaling Technology). Cells exposed to bleomycin (0.1 U/ml) for 1 h were used as a positive control. Immunoblots were developed using ECL HRP substrate (Advansta, Menlo Park, CA).

Flow cytometry

Cell death was studied by staining cells with an FITC Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher Scientific). Early apoptotic cells were identified as annexin V^+/ propidium iodide $(PI)^-$, late apoptotic cells as annexin V^+/PI^+, and necrotic cells as annexin V^-/PI^+. Cells were stained with MitoTracker Red CMXRos (Thermo Fisher Scientific) for mitochondrial transmembrane potential quantification according to the manufacturer's instructions. Data were analyzed using a Fortessa flow cytometer (Becton-Dickinson, San Jose, CA) and quantified with FlowJo software (Tree Star, San Carlos, CA).

XTT assay

BEAS-2B cells were seeded at 3000 cells per well in 96-well plates and exposed to increasing concentrations of HDM up to 30 h in the presence of XTT reagent (Cell Signaling Technology). XTT reagent is reduced to formazan dye in metabolically active cells, which can be quantified by measuring the absorbance at a wavelength of 450 nm.

Nitrite assay

Cellular nitrite levels were assayed using Measure-iT high-sensitivity nitrite assay kit (Invitrogen). BEAS-2B cells were plated on 48-well plates and exposed to increasing concentrations of HDM. As a positive control, cells were treated with *S*-nitrosoglutathione (Sigma-Aldrich), an NO donor, with or without pretreatment of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (Sigma-Aldrich), an NO scavenger that served as a negative control. Culture medium was collected after 6 h of HDM exposure and immediately assayed for nitrite levels. Fluorescence values were determined at 365/450 nm using a spectrofluorometer.

XO activity assay

XO activity level in cells exposed to HDM was measured using XO fluorometric assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Limulus *amebocyte lysate endotoxin assay*

Endotoxin levels in HDM was measured using Pierce *Limulus* amebocyte lysate chromogenic endotoxin quantitation kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

Statistical analysis

All data are expressed as mean ± SEM. Statistical comparison of multigroup data were analyzed by ANOVA followed by Tukey, Bonferroni, or

Dunnett post hoc analysis using GraphPad PRISM 6, with $p < 0.05$ considered significantly different.

Results

Aeroallergens induce DNA damage in human bronchial epithelial cells

To elucidate the genotoxic potential of aeroallergens, human bronchial epithelial BEAS-2B cells were exposed to increasing concentrations of HDM, CAE, ASP, RWE, or LPS. These aeroallergens are commonly used to induce experimental allergic asthma models (12). Alkaline comet assay is a well-established tool to quantify a broad spectrum of DNA lesions including single-strand break, alkali-sensitive sites, and abasic site. CometChip is a modified comet assay that allows high throughput and automated data analysis, as reported previously (9–11, 13).

We observed concentration-dependent increases in DNA damage in cells exposed to HDM, LPS, or CAE, but not to ASP or RWE (Fig. 1A). The level of DNA damage is indicated by the percentage of DNA in the tail, as fragmented DNA migrates faster than intact DNA. Fig. 1B shows representative images of comets from cells exposed to HDM. Notably, the level of damage induced by 100 μg HDM was similar to that induced by 100 μM H_2O_2, a highly genotoxic dose. These results indicate that HDM can directly induce DNA damage, in addition to its ability to activate immune responses in asthma.

HDM induces DNA DSBs in human bronchial epithelial cells

DSB is one of the most significant forms of damage to DNA, because it can lead to chromosomal translocations and even cell death if not repaired properly (14). Following the formation of a DSB, H2AX is phosphorylated to form γH2AX, marking nucleosomes along megabases of DNA surrounding the DSB sites. Using immunocytochemistry, γH2AX nuclear foci can serve as a signal indicating the presence of DNA DSBs (15). In this study, we show that HDM concentration dependently induced γH2AX foci as shown by increased immunofluorescence staining, which is consistent with the formation of DSBs (Fig. 1C, see inset for higher magnification). Quantification of cells with more than 10 foci per nucleus showed that up to 50% displayed a significant increase in the level of DSBs when exposed to 100 μg HDM for 6 h. We demonstrated that HDM aeroallergen can directly cause DNA DSBs in airway cells, even in the absence of immune cells that are known to secrete high levels of RONS.

HDM induces ROS production and increases mitochondrial oxidative stress in human bronchial epithelial cells

As compared with untreated controls, H_2O_2 (positive control) induced intracellular ROS (green signal) in BEAS-2B cells (Fig. 2A). HDM was able to trigger the production of ROS in a concentration-dependent manner, as shown by the significantly elevated green signal in cells. To learn more about the origins of ROS, we measured mitochondrial ROS using MitoTracker Red CM-H_2XRos, which stains the mitochondria in live cells upon oxidation (16). HDM increased mitochondrial oxidative stress in BEAS-2B cells, as shown by the enhanced red fluorescence signal observed (Fig. 2B).

HDM increases inducible NO synthase expression and nitrite secretion in bronchial epithelial cells

The endogenous production of NO by NO synthase (NOS) has been implicated as a pathophysiology event in asthma (17). NO has short half-life and converts rapidly to the physiologically stable nitrite (18). Hence, we measured the expression level of inducible NOS (iNOS) and the level of nitrite in cells. HDM induced significant increases in iNOS gene expression in BEAS-2B cells (Fig. 2C, top). In parallel, nitrite levels in BEAS-2B cells increased following exposure to increasing concentrations of HDM, although they did not reach a significant level (Fig. 2C, bottom).

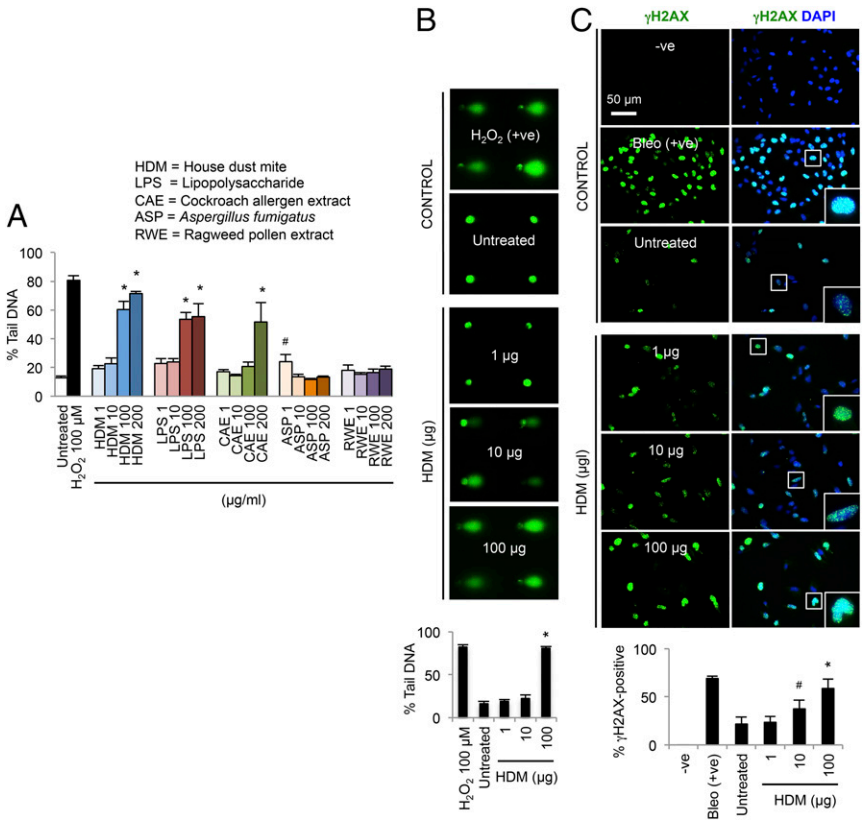

FIGURE 1. Aeroallergens induce DNA damage in human bronchial epithelial cells. (**A**) Cells were exposed to aeroallergens for 6 h and DNA damage was measured using alkaline comet assay. Percentage of DNA intensity in comet tail was quantified using in-house analysis software and plotted as shown. (**B**) Photomicrographs shown are representative images of comets from cells exposed to HDM (1, 10, 100 μg). Quantification of percentage of DNA in comet tails revealed the level of DNA damage after HDM exposure. (**C**) BEAS-2B cells were immunestained for DNA DSB marker, γH2AX (green) and counterstained with nuclei stain, DAPI (blue). Cells with ≥10 foci were counted as positive. Quantification was performed using ImageJ on 10 random images taken per set of treatment group per experiment. Original magnification ×400. All experiments were repeated at least three times and data presented are mean ± SEM. $^{*}p < 0.01$, $^{\#}p < 0.05$ indicate significant difference from untreated control.

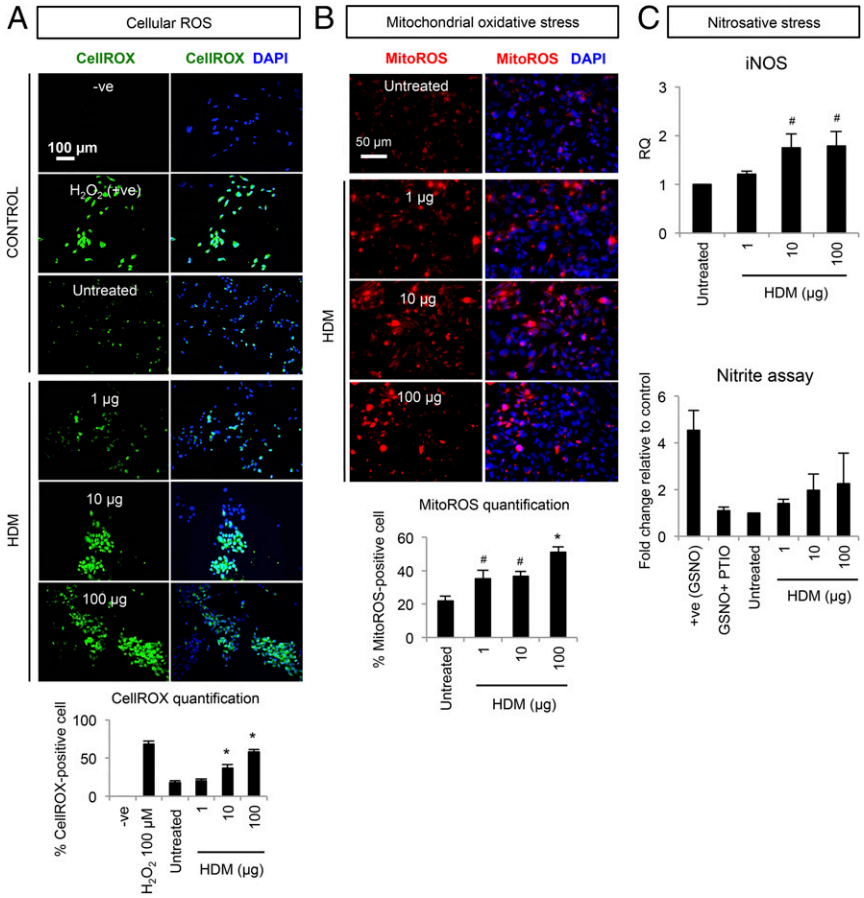

FIGURE 2. HDM triggers ROS production, and increases mitochondrial oxidative stress and nitrosative stress in cells. (**A**) After HDM exposure, cells were fixed and stained with CellROX to measure oxidative stress level. Green signals indicate the presence of oxidative stress in cells. Percentage of CellROX-positive cells was quantified on 10 random fields per treatment group. (**B**) Cells were stained with MitoTracker Red CM-H_2XRos to detect mitochondrial ROS (red). (**C**) iNOS gene expression in cells exposed to HDM for 6 h was measured with RT-PCR. Nitrite level in cell-culture medium was measured after cells were exposed to HDM for 6 h. All experiments were repeated at least three times and data presented are mean ± SEM. $^{*}p < 0.01$, $^{\#}p < 0.05$ indicate significant difference from untreated control.

HDM induces Nrf2 nuclear translocation and modulates antioxidant gene expression in bronchial epithelial cells

Nrf2 is a key regulatory transcription factor that induces antioxidant genes to protect against the deleterious effects of ROS (19). To study the impact of HDM in regulating antioxidant responses, we measured the activation of Nrf2 by quantifying its nuclear translocation. Immunoblotting of nuclear protein lysate reveals that HDM induced Nrf2 nuclear translocation in BEAS-2B cells after 6 h HDM exposure (Fig. 3A). Interestingly, at the same time point, we observed a reduction in antioxidant gene expression, indicating the oxidant-antioxidant balance was disrupted despite the rise in nuclear Nrf2 (Fig. 3B, left). To test if Nrf2 translocation leads to a delayed rescue response, we measured the antioxidant gene expression at 20 h post HDM exposure. The expression of many Nrf2-dependent genes including superoxide dismutase (SOD) 1, SOD3, GST, and glutathione-disulfide reductase returned to the basal level (Fig. 3B, right). More importantly, antioxidant genes SOD2, catalase, and NADPH dehydrogenase quinone 1 were all significantly elevated.

HDM reduces cell proliferation, induces AIF translocation, and causes cell death

To assess the impact of HDM on cell proliferation, an XTT assay was performed. Low-concentration HDM (1 and 10 µg) did not affect cell proliferation even after 30 h of incubation. However, high-concentration HDM (100 µg) reduced cell proliferation starting 8 h after exposure (Fig. 4A).

AIF is an intermembrane mitochondrial flavoprotein released from mitochondria into the cytosol during apoptosis. Translocation of AIF into the nucleus activates the caspase-independent cell death pathway (20). Immunofluorescence staining and quantification of AIF-positive nuclei showed a significant increase in the translocation of AIF (green signal) from the cytosol to the nucleus (blue) in bronchial epithelial cells exposed to HDM (10 and 100 µg) (Fig. 4B, see inset for higher magnification). This suggests that HDM exposure may activate the AIF-regulated cell death pathway in bronchial epithelial cells.

To quantify cell death, we stained cells with annexin V and PI. Cells exposed to HDM for 6 h showed slightly elevated apoptosis as compared with untreated control. Prolonged exposure to HDM for 20 h led to a significant increase in apoptosis (Fig. 4C). This suggests that the decrease in proliferation in cells with prolonged HDM exposure may be due to apoptosis.

Antioxidants suppress HDM-induced DNA damage

To study if antioxidants could prevent HDM-induced DNA damage in BEAS-2B cells, we coincubated cells with exogenous glutathione or catalase during HDM exposure. Catalase treatment significantly reduced intracellular ROS (green signal) in BEAS-2B cells exposed to 100 µg HDM (Fig. 5A). In addition, we observed a significant concentration-dependent reduction in HDM-induced DNA damage in cells coincubated with antioxidants, measured using an alkaline comet assay (Fig. 5B). This suggests that HDM-induced DNA damage can be prevented by antioxidant treatment, revealing a novel role of antioxidants in genome protection.

HDM induces NOX gene expression

Airway epithelial cells express several NOX isoforms, which each play a different role in host defense, cell proliferation, and differentiation (7, 21, 22). To understand if NOX-derived ROS plays a role in HDM-induced cellular injury, NOX gene expression was measured in BEAS-2B cells exposed to HDM. HDM significantly induced NOX2 and NOX4 gene expression in bronchial epithelial

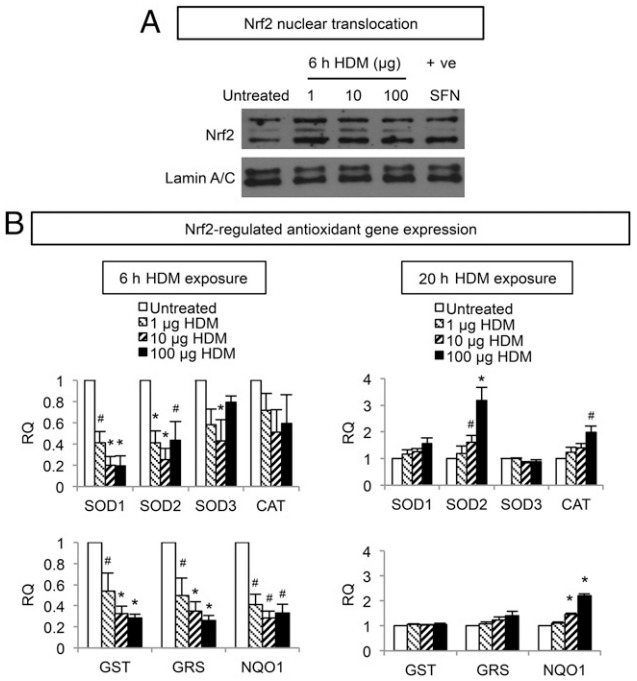

FIGURE 3. HDM alters antioxidant gene expression. (**A**) Nuclear Nrf2 level was determined using immunoblotting. Cells treated with sulforaphane (SFN) were used as positive control. (**B**) RNA extraction was performed and antioxidant gene expression was measured using RT-PCR in cells exposed to HDM for 6 (left) or 20 h (right). All experiments were repeated at least three times and data presented are mean ± SEM. $^{*}p < 0.01$, $^{\#}p < 0.05$ indicate significant difference from untreated control.

cells (Fig. 6A). NOX2 and NOX4 have been shown to play a crucial role in asthma pathogenesis (23–26). Most importantly, the NOX inhibitor apocynin significantly reduced HDM-induced DNA DSBs in the bronchial epithelial cells, as shown by γH2AX immunostaining (Fig. 6C), suggesting that HDM-induced DNA damage is NOX dependent.

In contrast, XO, another ubiquitous enzyme responsible for ROS and NO production (27), was not significantly activated by HDM exposure (Fig. 6B). Consistent with the finding, oxypurinol, an XO inhibitor, was not able to reduce HDM-induced DSBs (Fig. 6C). Our data suggest that HDM exposure induced NOX2 and NOX4 expressions but not XO activity, providing some mechanistic insights into HDM-induced oxidative stress and the ensuing DNA damaging effects.

Discussion

Exposure to HDM allergen is a major risk factor for allergic sensitization and asthma development (28, 29). Between 50 and 85% of asthmatics are found to be allergic to HDM (30, 31). HDM is a complex biological allergen that displays strong allergenic potential, mainly through its ability to interact with the innate immune system (31, 32). The airway epithelium is the first line of defense for the lung tissue when exposed to environmental stimuli including aeroallergens. It plays a central role in activating the immune response toward external stimuli (33). In this study, we revealed that HDM could directly induce DNA damage in BEAS-2B cells by triggering RONS formation, probably via enhanced NOX gene expression and the weakening of antioxidant responses. It can also reduce cell proliferation and induce cell death, potentially dampening the ability of cells to regenerate. We postulate that the DNA-damaging potential of aeroallergens on the bronchial epithelial cells plays a contributing role in asthma pathogenesis. This study suggests that allergens that are generally perceived as being harmless cause more damage than previously thought.

It has been shown that HDM activates bronchial epithelial cells and releases pro-Th2 cytokines such as GM-CSF, TSLP, IL-25, and IL-33 (33). One possible path is through its pathogen-associated molecular patterns that can be recognized by pattern recognition receptors expressed on the apical surface of airway epithelial cells. Proteolytic cleavage of host proteins by the protease activity of allergens, tissue damage, and metabolic changes induced by allergens can also drive Th2 responses in cells (34). In addition, allergen-induced tissue damage releases damage-associated molecular patterns such as ATP and uric acid, which serve as danger signals that alert the immune system to tissue damage (5).

Increased ROS has been associated with allergic lung inflammation, lung injury, and vascular remodeling. We observed elevated levels of ROS when BEAS-2B cells were exposed to HDM. Some allergens contain NOX and can induce oxidative stress in the airway epithelium (35, 36), pointing to the possibility that HDM-induced oxidative stress may also be dependent on NOX. In fact, studies have shown that NOX regulates various phases of asthmatic

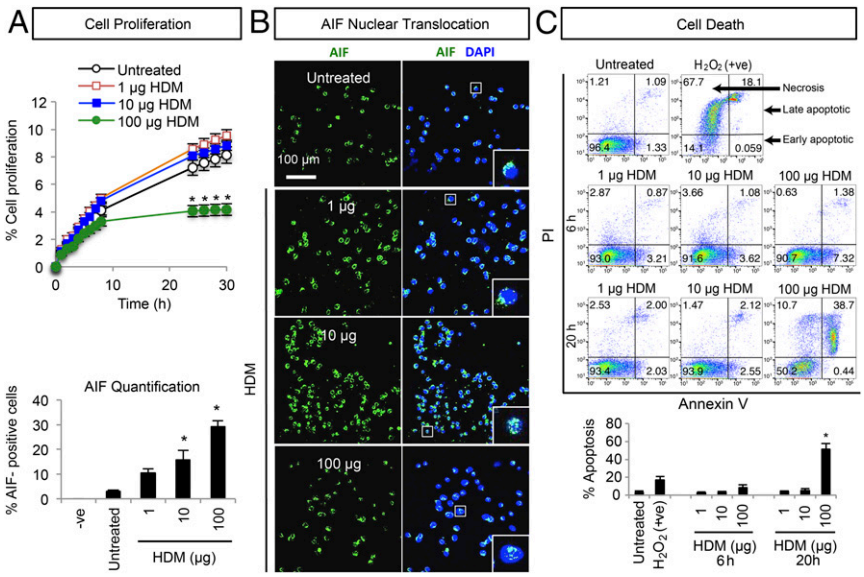

FIGURE 4. HDM reduces cell proliferation, and induces AIF nuclear translocation and cell death. (**A**) Cell proliferation was determined using XTT assay, up to 30 h after HDM exposure. (**B**) Cells were stained with AIF (green) and DAPI (blue). Colocalization of AIF with DAPI was quantified. (**C**) Cell death assay was performed using annexin V/PI staining. Cells positive for annexin V only or both annexin V and PI were considered as early apoptotic and late apoptotic cells, respectively. Data were collected using flow cytometry, and analyzed using FlowJo software. All experiments were repeated at least three times and data presented are mean ± SEM. $^{*}p < 0.01$, $^{\#}p < 0.05$ indicate significant difference from untreated control.

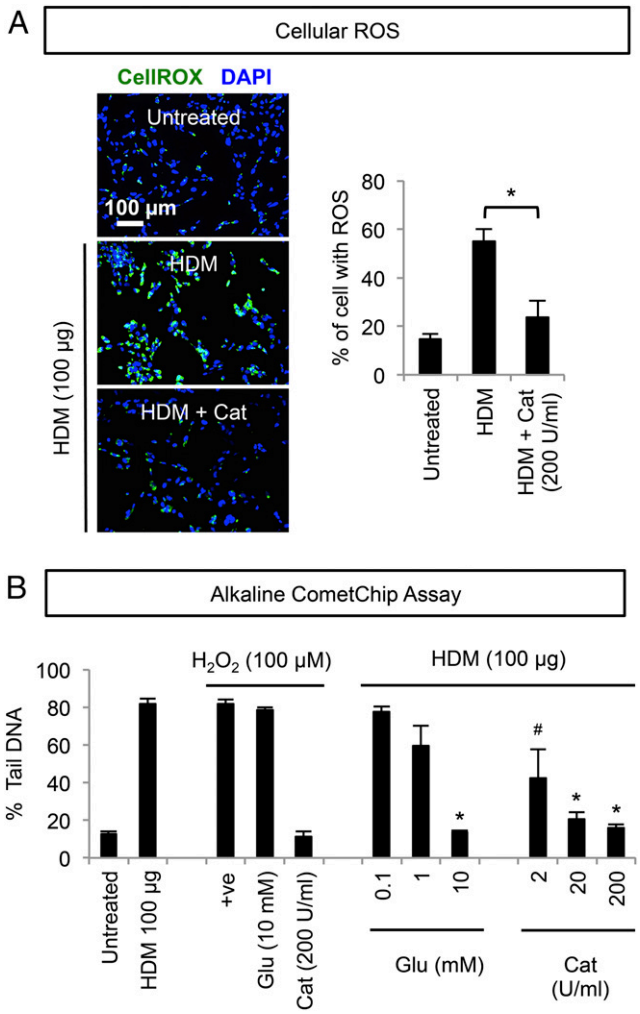

FIGURE 5. HDM-induced DNA damage can be prevented by antioxidant treatment. BEAS-2B cells were exposed to HDM for 6 h with or without antioxidant glutathione and catalase treatment, followed by CellROX staining and alkaline comet assay. (**A**) Green signal (CellROX) indicates the presence of intracellular ROS in cells. (**B**) DNA damage in cells is indicated by the percentage of DNA in the comet tail. Experiments were repeated at least three times and mean score ± SEM was plotted. $^{*}p < 0.01$, $^{\#}p < 0.05$ indicate significant difference from untreated control.

pathogenesis (25, 26, 37). For instance, NOX4 overexpression in asthma has been associated with increased oxidative stress and agonist-induced airway smooth muscle hyper-contractility (25). Asthmatic bronchial epithelial cells that overexpressed NOX4 and NOX dual oxidase 1 and 2 were associated with epithelial ciliary dysfunction (26). NOX2 enhanced eosinophil recruitment and promoted allergic asthma airway inflammation (23, 24). The NOX inhibitor apocynin has been shown to suppress the production of TNF-α, IL-1β, and IL-6, as well as airway hyperresponsiveness and airway inflammation in OVA-induced experimental asthma (38). In this study, although we have shown that HDM induced NOX2 and NOX4 gene expression, we did not observe a dose-dependent effect. Hence, it is noteworthy to mention that NOX is not the only mechanism that leads to free radical production. We have shown that HDM dysregulated the mitochondrial electron transport chain (another significant source of ROS) in BEAS-2B cells, as suggested by the elevated mitochondrial oxidative stress and weakened Nrf2-dependent antioxidant gene expression. These could be the alternative mechanisms that lead to the increased oxidative stress in BEAS-2B cells, especially in the higher concentrations of HDM (at 10 and 100 μg) exposure. Nevertheless, our studies highlighted the importance of NOX enzymes, especially NOX2 and NOX4, in HDM-induced epithelial cell DNA damage, probably via ROS production.

It has been shown that RWE can cause oxidative damage to mitochondrial respiratory proteins and triggers ROS production from the mitochondrial respiratory chain complex in human airway epithelial cells (39). Oxidative stress as a result of mitochondrial dysfunction has been associated with the development of allergic airway inflammation and airway remodeling (40, 41). In line with these findings, we showed in this study that HDM increases mitochondrial ROS in BEAS-2B cells. $\cdot O_2^-$ is the main damaging by-product of mitochondrial oxidative phosphorylation, and resides on the matrix side of the inner mitochondrial membrane (42). This may account for the strong oxidation potential within mitochondria as shown in our results.

Our results further implicate the potential role of mitochondrial oxidative stress that leads to AIF translocation and ultimately the activation of the cell death pathway. Under metabolic stress, mitochondria-derived oxidants function as signaling molecules, such as in apoptotic pathways (43). The impaired mitochondrial respiratory chain function affects ATP production and impacts cell viability (44). Mitochondrial ROS mediates the release of proapoptotic cytochrome c and AIF into the cytosol, leading to the activation of caspase-dependent and caspase-independent cell death pathways, respectively (44, 45). The release of AIF from mitochondria into the cytosol can also be attributed to oxidative modification by mitochondrial ROS that has changed its conformation (45). In this study, after HDM exposure we observed a significant increase in the translocation of AIF into the nucleus, which could then trigger caspase-independent cell death. In parallel, we detected reduced cell proliferation and increased cell death. These are likely to be the consequence of the activation of mitochondria-dependent cell death. Notably, AIF nuclear translocation has been related to cell death by necroptosis, a form of programmed necrosis (46). Interestingly, in our studies, HDM does not induce necrosis (Supplemental Fig. 1), suggesting HDM-induced cell death is mainly through apoptosis. In the asthmatic airway, extensive bronchial epithelial cell death has been observed (47, 48). Failure to regenerate to replace damaged bronchial epithelial cells could result in the disruption of the lung barrier integrity and increased susceptibility to lung injury.

The Nrf2 transcription factor provides an earliest antioxidant defense and cytoprotection from oxidative damage by inducing the expression of antioxidant genes (49). In animal models, disruption of Nrf2 enhances the susceptibility to airway inflammation and exacerbates allergic inflammation induced by allergens (50). We observed an increase in the translocation of Nrf2 transcription factor into the nucleus, suggesting an immediate and first-line oxidative stress response in cells exposed to HDM. Indeed, antioxidant gene expression was dampened shortly after HDM exposure. This observation is consistent with other reports that suggest there is a defect in antioxidant capacity in asthmatic lungs (51–53). Nevertheless, increased antioxidant expression was evident after 20 h of HDM exposure. This is likely to result from the enhanced Nrf2 nuclear translocation observed at 6 h post HDM exposure to restore the oxidant-antioxidant balance in cells. Notably, SOD2 gene expression was significantly elevated after 20 h HDM exposure. SOD2 is a mitochondrial matrix enzyme that scavenges oxygen radicals produced by mitochondrial during electron transport reactions (54). This suggests the presence of mitochondrial oxidative stress in cells, hence requiring constant expression of SOD2.

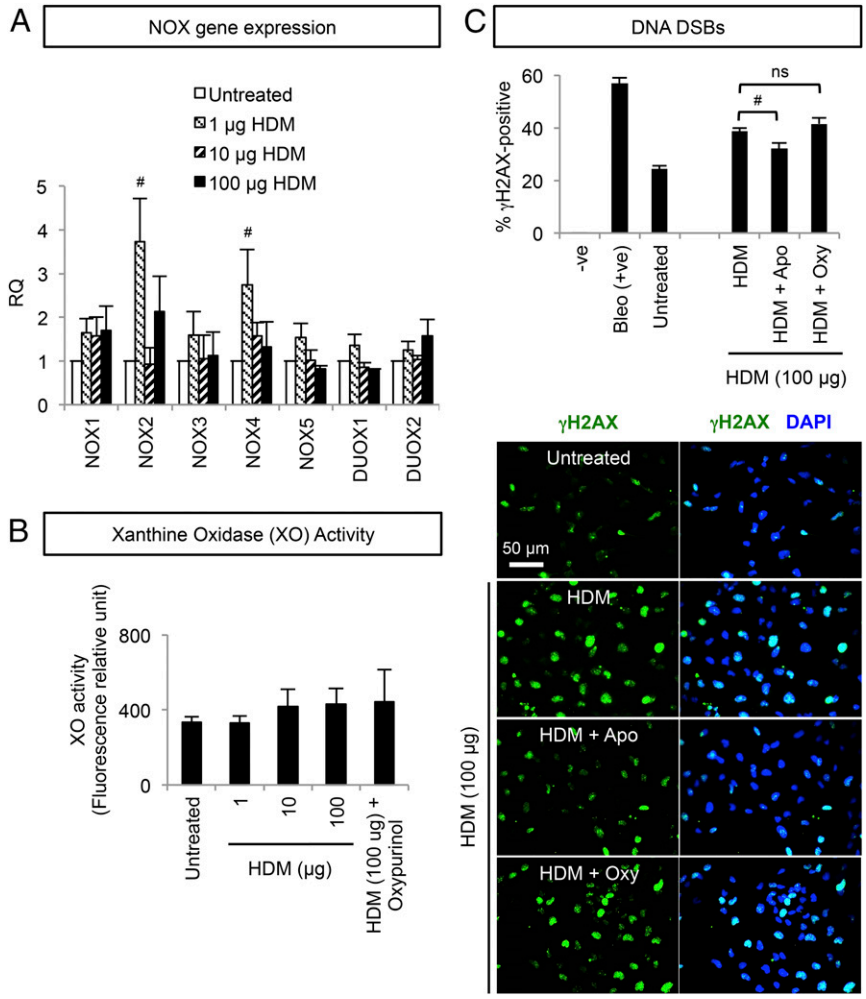

FIGURE 6. HDM-induced DSBs are NOX dependent. (**A**) NOX gene expression was measured using RT-PCR in BEAS-2B exposed to HDM for 6 h. (**B**) XO activity was quantified using ELISA. (**C**) To study the effects of NOX inhibitor apocynin (Apo) and XO inhibitor oxypurinol (Oxy) in HDM-induced DNA DSBs, cells were pretreated with inhibitors for 1 h before coincubating with 100 μg HDM for 6 h. Cells were stained with γH2AX (green) and counterstained with nuclei stain, DAPI (blue). Cells with ≥10 foci were counted as positive. Quantification was performed using ImageJ on 10–13 random images taken per set of treatment group per experiment. Original magnification ×400. All experiments were repeated at least three times and data presented are mean ± SEM. $^{\#}p < 0.05$ indicates significant difference from untreated control or respective HDM control. n.s., no significant difference.

In this study, we have shown that HDM activates pathways such as iNOS expression, nitrite level, Nrf2 translocation, and apoptosis. However, it is important to note that these pathways could also be mediated directly by RONS. For instance, iNOS expression is NOX and RONS dependent (55, 56) and RONS-induced oxidative stress can trigger Nrf2 nuclear translocation (57). In addition, ROS is an important regulator of apoptotic cell death, which could be induced by excessive DNA damage (58, 59).

To investigate the potential of antioxidants such as glutathione and catalase in protection against HDM-induced DNA damage, we added glutathione and catalase as supplements into culture medium. Glutathione is a thiol compound and is a major antioxidant present in cells to maintain a tight control of the redox status (60). In a reduced state, glutathione scavenges a wide variety of ROS, including $\cdot O_2^-$, hydroxyl radical ($\cdot OH^-$), protein and DNA radicals, by donating electrons and being oxidized to a glutathionethiyl radical (61). Although catalase is commonly known an intracellular antioxidant enzyme that catalyzes the reaction that converts H_2O_2 to water and oxygen, it has been shown that catalase is able to scavenge H_2O_2 in extracellular compartments (62, 63). Replenishing of glutathione and catalase pools in cells scavenges and prevents the accumulation of ROS, and as a result, reduces DNA damage in cells. Indeed, our findings demonstrated the ability of exogenous glutathione and catalase to reduce HDM-induced DNA damage in BEAS-2B cells. Interestingly, although both catalase and glutathione were able to protect cells from HDM-induced DNA damage, only catalase was able to prevent H_2O_2-induced DNA damage. This suggests that the two antioxidants possess different signaling mechanisms in scavenging ROS. Nevertheless, this highlights the importance and ability of exogenous antioxidants to protect lung cells from the genotoxic effects of aeroallergens, revealing a novel application of antioxidants for treating asthma.

It has been shown than HDM binds to TLR-4 on lung epithelial cells to induce asthma (64). To investigate the role of TLR-4 in HDM-induced DNA damage in bronchial epithelial cells, we employed a TLR-4 specific agonist and antagonist to activate or block the binding of HDM to TLR-4 on bronchial epithelial cells, respectively. We have shown that HDM-induced DNA damage is independent of TLR-4 activation (Supplemental Fig. 2).

In addition, to determine whether the DNA damage observed in this study was induced by an endotoxin contaminant in HDM extracts, we first measured the endotoxin level in HDM extracts using the *Limulus* amebocyte lysate endotoxin assay. The result showed that 1 μg HDM contains 6.51391 ± 0.053805 endotoxin units (equivalent to 0.00651 μg LPS) (Supplemental Fig. 3A). It can be deduced that 100 μg HDM consists of ~651 endotoxin units (equivalent to 0.651 μg LPS). We exposed cells to LPS (1 or 10 μg/ml) for 6 h and measured DNA DSBs using γH2AX immunofluorescence staining. We showed that the levels of DNA DSBs in the presence of 1 or 10 μg/ml LPS are similar to the level in untreated cells (Supplemental Fig. 3B). This suggests that HDM-induced DNA DSBs in BEAS-2B cells are mainly contributed by HDM itself. Moreover, to rule out the possibility of the DNA contaminants in HDM extracts contributing to the damaged DNA observed in this study, we probed for γH2AX protein levels

in HDM extract lysates (1, 10, and 100 μg) using immunoblotting (Supplemental Fig. 3C). No γH2AX bands were detected in HDM extracts, suggesting that γH2AX observed in HDM-challenged BEAS-2B was from the bronchial epithelial cells and not the HDM extracts.

Excessive or irreparable DNA damage activates cell death pathways, and in asthma, airway epithelial cell death accounts for excessive epithelial loss, which is one of the contributing mechanisms for worsening symptoms in allergic asthma. In this study, we have shown that HDM allergen is genotoxic to bronchial epithelial cells and affects cell proliferation. The bronchial epithelium, which suffers from genotoxicity and cytotoxicity, could be compromised structurally and functionally. This is an important yet understudied mechanism that drives asthma pathogenesis.

To our knowledge, the current study revealed for the first time that HDM induces DNA damage in bronchial epithelial cells by triggering the production of genotoxic RONS. HDM exposure increases cellular oxidative and nitrosative stress, induces NOX2 and NOX4 gene expression, and alters Nrf2-dependent antioxidant responses. In addition, HDM reduces cell proliferation and induces cell death, which could affect airway epithelium regeneration after cell injury as a result of allergen exposure. Further, antioxidant and NOX inhibitor treatments are able to protect cells from HDM-induced DNA damage, indicating that RONS are a major source of DNA damage in cells exposed to HDM. Although further studies are needed to ascertain the specific mechanism of how HDM leads to an increase in ROS in cells, our study points to the possibility that inhibition of oxidative stress or targeting oxidative mechanisms that cause epithelial cell injury could alter asthma pathophysiology.

Acknowledgments

We are grateful to Prof. Bevin P. Engelward from the Department of Biological Engineering, Massachusetts Institute of Technology for sharing expertise and providing technical assistance in CometChip assay.

Disclosures

The authors have no financial conflicts of interest.

References

1. Pawankar, R., G. W. Canonica, S. T. Holgate, and R. F. Lockey, eds. 2011. *World Allergy Organization (WAO) White Book on Allergy.* Vol. 3. World Allergy Organization, Milwaukee, WI.
2. Galli, S. J., M. Tsai, and A. M. Piliponsky. 2008. The development of allergic inflammation. *Nature* 454: 445–454.
3. Trompette, A., S. Divanovic, A. Visintin, C. Blanchard, R. S. Hegde, R. Madan, P. S. Thorne, M. Wills-Karp, T. L. Gioannini, J. P. Weiss, and C. L. Karp. 2009. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 457: 585–588.
4. Lambrecht, B. N., and H. Hammad. 2014. Allergens and the airway epithelium response: gateway to allergic sensitization. *J. Allergy Clin. Immunol.* 134: 499–507.
5. Lambrecht, B. N., and H. Hammad. 2012. The airway epithelium in asthma. *Nat. Med.* 18: 684–692.
6. Holgate, S. T., P. Lackie, S. Wilson, W. Roche, and D. Davies. 2000. Bronchial epithelium as a key regulator of airway allergen sensitization and remodeling in asthma. *Am. J. Respir. Crit. Care Med.* 162: S113–S117.
7. Panday, A., M. K. Sahoo, D. Osorio, and S. Batra. 2015. NADPH oxidases: an overview from structure to innate immunity-associated pathologies. *Cell. Mol. Immunol.* 12: 5–23.
8. Soria, G., S. E. Polo, and G. Almouzni. 2012. Prime, repair, restore: the active role of chromatin in the DNA damage response. *Mol. Cell* 46: 722–734.
9. Ge, J., D. N. Chow, J. L. Fessler, D. M. Weingeist, D. K. Wood, and B. P. Engelward. 2015. Micropatterned comet assay enables high throughput and sensitive DNA damage quantification. *Mutagenesis* 30: 11–19.
10. Wood, D. K., D. M. Weingeist, S. N. Bhatia, and B. P. Engelward. 2010. Single cell trapping and DNA damage analysis using microwell arrays. *Proc. Natl. Acad. Sci. USA* 107: 10008–10013.
11. Weingeist, D. M., J. Ge, D. K. Wood, J. T. Mutamba, Q. Huang, E. A. Rowland, M. B. Yaffe, S. Floyd, and B. P. Engelward. 2013. Single-cell microarray enables high-throughput evaluation of DNA double-strand breaks and DNA repair inhibitors. *Cell Cycle* 12: 907–915.
12. Meyer-Martin, H., S. Reuter, and C. Taube. 2014. Mouse models of allergic airway disease. *Methods Mol. Biol.* 1193: 127–141.
13. Ge, J., S. Prasongtanakij, D. K. Wood, D. M. Weingeist, J. Fessler, P. Navasummrit, M. Ruchirawat, and B. P. Engelward. 2014. CometChip: a high-throughput 96-well platform for measuring DNA damage in microarrayed human cells. *J. Vis. Exp.* 92: e50607.
14. Jeggo, P. A., and M. Löbrich. 2007. DNA double-strand breaks: their cellular and clinical impact? *Oncogene* 26: 7717–7719.
15. Chapman, J. R., M. R. Taylor, and S. J. Boulton. 2012. Playing the end game: DNA double-strand break repair pathway choice. *Mol. Cell* 47: 497–510.
16. Lambeth, J. D. 2004. NOX enzymes and the biology of reactive oxygen. *Nat. Rev. Immunol.* 4: 181–189.
17. Prado, C. M., M. A. Martins, and I. F. Tibério. 2011. Nitric oxide in asthma physiopathology. *ISRN Allergy* 2011: 832560.
18. Shiva, S. 2013. Nitrite: a physiological store of nitric oxide and modulator of mitochondrial function. *Redox Biol.* 1: 40–44.
19. Sporn, M. B., and K. T. Liby. 2012. NRF2 and cancer: the good, the bad and the importance of context. *Nat. Rev. Cancer* 12: 564–571.
20. Daugas, E., S. A. Susin, N. Zamzami, K. F. Ferri, T. Irinopoulou, N. Larochette, M. C. Prévost, B. Leber, D. Andrews, J. Penninger, and G. Kroemer. 2000. Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *FASEB J.* 14: 729–739.
21. Forteza, R., M. Salathe, F. Miot, R. Forteza, and G. E. Conner. 2005. Regulated hydrogen peroxide production by Duox in human airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 32: 462–469.
22. Nagai, K., T. Betsuyaku, M. Suzuki, Y. Nasuhara, K. Kaga, S. Kondo, and M. Nishimura. 2008. Dual oxidase 1 and 2 expression in airway epithelium of smokers and patients with mild/moderate chronic obstructive pulmonary disease. *Antioxid. Redox Signal.* 10: 705–714.
23. Abdala-Valencia, H., J. Earwood, S. Bansal, M. Jansen, G. Babcock, B. Garvy, M. Wills-Karp, and J. M. Cook-Mills. 2007. Nonhematopoietic NADPH oxidase regulation of lung eosinophilia and airway hyperresponsiveness in experimentally induced asthma. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 292: L1111–L1125.
24. Cook-Mills, J. M., M. E. Marchese, and H. Abdala-Valencia. 2011. Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants. *Antioxid. Redox Signal.* 15: 1607–1638.
25. Sutcliffe, A., F. Hollins, E. Gomez, R. Saunders, C. Doe, M. Cooke, R. A. Challiss, and C. E. Brightling. 2012. Increased nicotinamide adenine dinucleotide phosphate oxidase 4 expression mediates intrinsic airway smooth muscle hypercontractility in asthma. *Am. J. Respir. Crit. Care Med.* 185: 267–274.
26. Wan, W. Y., F. Hollins, L. Haste, L. Woodman, R. A. Hirst, S. Bolton, E. Gomez, A. Sutcliffe, D. Desai, L. Chachi, et al. 2016. NADPH oxidase-4 overexpression is associated with epithelial ciliary dysfunction in neutrophilic asthma. *Chest* 149: 1445–1459.
27. Li, H., A. Samouilov, X. Liu, and J. L. Zweier. 2001. Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrite reduction. Evaluation of its role in nitric oxide generation in anoxic tissues. *J. Biol. Chem.* 276: 24482–24489.
28. Arbes Jr., S. J., R. D. Cohn, M. Yin, M. L. Muilenberg, H. A. Burge, W. Friedman, and D. C. Zeldin. 2003. House dust mite allergen in US beds: results from the First National Survey of Lead and Allergens in Housing. *J. Allergy Clin. Immunol.* 111: 408–414.
29. Hales, B. J., A. C. Martin, L. J. Pearce, I. A. Laing, C. M. Hayden, J. Goldblatt, P. N. Le Souëf, and W. R. Thomas. 2006. IgE and IgG anti-house dust mite specificities in allergic disease. *J. Allergy Clin. Immunol.* 118: 361–367.
30. Thomas, W. R., W. A. Smith, B. J. Hales, K. L. Mills, and R. M. O'Brien. 2002. Characterization and immunobiology of house dust mite allergens. *Int. Arch. Allergy Immunol.* 129: 1–18.
31. Gregory, L. G., and C. M. Lloyd. 2011. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol.* 32: 402–411.
32. Fahlbusch, B., A. Koch, J. Douwes, W. Bischof, U. Gehring, K. Richter, H. E. Wichmann, and J. Heinrich. 2003. The effect of storage on allergen and microbial agent levels in frozen house dust. *Allergy* 58: 150–153.
33. Lambrecht, B. N., and H. Hammad. 2015. The immunology of asthma. *Nat. Immunol.* 16: 45–56.
34. Post, S., M. C. Nawijn, T. L. Hackett, M. Baranowska, R. Gras, A. J. van Oosterhout, and I. H. Heijink. 2012. The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. *Thorax* 67: 488–495.
35. Bacsi, A., N. Dharajiya, B. K. Choudhury, S. Sur, and I. Boldogh. 2005. Effect of pollen-mediated oxidative stress on immediate hypersensitivity reactions and late-phase inflammation in allergic conjunctivitis. *J. Allergy Clin. Immunol.* 116: 836–843.
36. Boldogh, I., A. Bacsi, B. K. Choudhury, N. Dharajiya, R. Alam, T. K. Hazra, S. Mitra, R. M. Goldblum, and S. Sur. 2005. ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation. *J. Clin. Invest.* 115: 2169–2179.
37. van der Vliet, A. 2011. Nox enzymes in allergic airway inflammation. *Biochim. Biophys. Acta* 1810: 1035–1044.
38. Kim, S. Y., K. A. Moon, H. Y. Jo, S. Jeong, S. H. Seon, E. Jung, Y. S. Cho, E. Chun, and K. Y. Lee. 2012. Anti-inflammatory effects of apocynin, an inhibitor of NADPH oxidase, in airway inflammation. *Immunol. Cell Biol.* 90: 441–448.
39. Aguilera-Aguirre, L., A. Bacsi, A. Saavedra-Molina, A. Kurosky, S. Sur, and I. Boldogh. 2009. Mitochondrial dysfunction increases allergic airway inflammation. *J. Immunol.* 183: 5379–5387.

40. Mabalirajan, U., A. K. Dinda, S. Kumar, R. Roshan, P. Gupta, S. K. Sharma, and B. Ghosh. 2008. Mitochondrial structural changes and dysfunction are associated with experimental allergic asthma. *J. Immunol.* 181: 3540–3548.
41. Kim, S. R., D. I. Kim, S. H. Kim, H. Lee, K. S. Lee, S. H. Cho, and Y. C. Lee. 2014. NLRP3 inflammasome activation by mitochondrial ROS in bronchial epithelial cells is required for allergic inflammation. *Cell Death Dis.* 5: e1498.
42. Ott, M., V. Gogvadze, S. Orrenius, and B. Zhivotovsky. 2007. Mitochondria, oxidative stress and cell death. *Apoptosis* 12: 913–922.
43. Finkel, T., and N. J. Holbrook. 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239–247.
44. Fariss, M. W., C. B. Chan, M. Patel, B. Van Houten, and S. Orrenius. 2005. Role of mitochondria in toxic oxidative stress. *Mol. Interv.* 5: 94–111.
45. Norberg, E., S. Orrenius, and B. Zhivotovsky. 2010. Mitochondrial regulation of cell death: processing of apoptosis-inducing factor (AIF). *Biochem. Biophys. Res. Commun.* 396: 95–100.
46. Galluzzi, L., and G. Kroemer. 2008. Necroptosis: a specialized pathway of programmed necrosis. *Cell* 135: 1161–1163.
47. Erle, D. J., and D. Sheppard. 2014. The cell biology of asthma. *J. Cell Biol.* 205: 621–631.
48. Zhou, C., G. Yin, J. Liu, X. Liu, and S. Zhao. 2011. Epithelial apoptosis and loss in airways of children with asthma. *J. Asthma* 48: 358–365.
49. Gorrini, C., I. S. Harris, and T. W. Mak. 2013. Modulation of oxidative stress as an anticancer strategy. *Nat. Rev. Drug Discov.* 12: 931–947.
50. Li, Y. J., T. Kawada, and A. Azuma. 2013. Nrf2 is a protective factor against oxidative stresses induced by diesel exhaust particle in allergic asthma. *Oxid. Med. Cell. Longev.* 2013: 323607.
51. Corradi, M., G. Folesani, R. Andreoli, P. Manini, A. Bodini, G. Piacentini, S. Carraro, S. Zanconato, and E. Baraldi. 2003. Aldehydes and glutathione in exhaled breath condensate of children with asthma exacerbation. *Am. J. Respir. Crit. Care Med.* 167: 395–399.
52. De Raeve, H. R., F. B. Thunnissen, F. T. Kaneko, F. H. Guo, M. Lewis, M. S. Kavuru, M. Secic, M. J. Thomassen, and S. C. Erzurum. 1997. Decreased Cu,Zn-SOD activity in asthmatic airway epithelium: correction by inhaled corticosteroid in vivo. *Am. J. Physiol.* 272: L148–L154.
53. Dworski, R. 2000. Oxidant stress in asthma. *Thorax* 55(Suppl. 2): S51–S53.
54. Wallace, D. C. 2012. Mitochondria and cancer. *Nat. Rev. Cancer* 12: 685–698.
55. Wu, F., K. Tyml, and J. X. Wilson. 2008. iNOS expression requires NADPH oxidase-dependent redox signaling in microvascular endothelial cells. *J. Cell. Physiol.* 217: 207–214.
56. Sun, J., L. J. Druhan, and J. L. Zweier. 2010. Reactive oxygen and nitrogen species regulate inducible nitric oxide synthase function shifting the balance of nitric oxide and superoxide production. *Arch. Biochem. Biophys.* 494: 130–137.
57. Nguyen, T., P. Nioi, and C. B. Pickett. 2009. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J. Biol. Chem.* 284: 13291–13295.
58. Circu, M. L., and T. Y. Aw. 2010. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic. Biol. Med.* 48: 749–762.
59. Kang, M. A., E. Y. So, A. L. Simons, D. R. Spitz, and T. Ouchi. 2012. DNA damage induces reactive oxygen species generation through the H2AX-Nox1/Rac1 pathway. *Cell Death Dis.* 3: e249.
60. Fitzpatrick, A. M., D. P. Jones, and L. A. Brown. 2012. Glutathione redox control of asthma: from molecular mechanisms to therapeutic opportunities. *Antioxid. Redox Signal.* 17: 375–408.
61. Franco, R., and J. A. Cidlowski. 2009. Apoptosis and glutathione: beyond an antioxidant. *Cell Death Differ.* 16: 1303–1314.
62. Goyal, M. M., and A. Basak. 2010. Human catalase: looking for complete identity. *Protein Cell* 1: 888–897.
63. Preston, T. J., W. J. Muller, and G. Singh. 2001. Scavenging of extracellular H2O2 by catalase inhibits the proliferation of HER-2/Neu-transformed rat-1 fibroblasts through the induction of a stress response. *J. Biol. Chem.* 276: 9558–9564.
64. Hammad, H., M. Chieppa, F. Perros, M. A. Willart, R. N. Germain, and B. N. Lambrecht. 2009. House dust mite allergen induces asthma via toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* 15: 410–416.