Acetaminophen Attenuates House Dust Mite–Induced Allergic Airway Disease in Mice

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ABSTRACT

Epidemiologic evidence suggests that N-acetyl-para-aminophenol (APAP) may play a role in the pathogenesis of asthma, likely through pro-oxidant mechanisms. However, no studies have investigated the direct effects of APAP on the development of allergic inflammation. To determine the likelihood of a causal relationship between APAP and asthma pathogenesis, we explored the effects of APAP on inflammatory responses in a murine house dust mite (HDM) model of allergic airway disease. We hypothesized that APAP would enhance the development of HDM-induced allergic inflammation. The HDM model consisted of once daily intranasal instillations for up to 2 weeks with HDM or vehicle administration 1 hour prior to HDM during either week 1 or 2. Primary assessment of inflammation included bronchoalveolar lavage (BAL), cytokine expression in lung tissue, and histopathology. Contrary to our hypothesis, the effects of HDM treatment were substantially diminished in APAP-treated groups compared with controls. APAP-treated groups had markedly reduced airway inflammation: including decreased inflammatory cells in the BAL fluid, lower cytokine expression in lung tissue, and less perivascular and peribronchiolar immune cell infiltration. The anti-inflammatory effect of APAP was not abrogated by an inhibitor of cytochrome P450 (P450) metabolism, suggesting that the effect was due to the parent compound or a non-P450 generated metabolite. Taken together, our studies do not support the biologic plausibility of the APAP hypothesis that APAP use may contribute to the causation of asthma. Importantly, we suggest the mechanism by which APAP modulates airway inflammation may provide novel therapeutic targets for asthma.

Introduction

The past three decades have witnessed a worldwide epidemic-scale increase in asthma prevalence, the cause of which is unknown. The increase in asthma prevalence occurred concomitantly with the expansion in acetaminophen [N-acetyl-para-aminophenol (APAP)] use following identification, in the early 1980s, of a suspected Reyes Syndrome risk from aspirin. The association between APAP use and asthma has prompted the APAP hypothesis that APAP contributes to the increase in asthma, likely through its pro-oxidant effects. Multiple large epidemiologic studies have found a strong association between APAP use and asthma prevalence in children and adults (Barr et al., 2004; Etminan et al., 2009; Beasley et al., 2011). However, due to inherent limitations of epidemiologic data, a causal relationship between APAP and asthma has not been established. One such limitation is the possibility of confounding by indication; for example, APAP is often used to treat fever caused by infections, and certain respiratory tract infections are independent risk factors for asthma (Heintze and Petersen, 2013). Supporting this explanation are several studies that found adjusting for respiratory infections diminished the association (Lowe et al., 2010; Sordillo et al., 2015).

Despite evidence supporting confounding factors, uncertainty and the near-ubiquitous use of APAP make the APAP–asthma association worthy of further investigation. The most developed of several proposed mechanisms for the association is that APAP-induced oxidative stress promotes the development of an asthmatic phenotype. Oxidative stress is a hallmark of asthma and is thought to be important in asthma pathogenesis. Markers of oxidant levels are higher in people with asthma, and polymorphisms in antioxidant genes such as glutathione (GSH) transferase are asthma risk factors (Riedl and Nel, 2008; Holguin, 2013). However, asthma is an inflammatory disease and oxidative stress may be a result as well as a cause of active airway inflammation, making full definition of its role difficult (Henricks and Nijkamp, 2001; Andreadis et al., 2003; Bhalla et al., 2009). Animal studies confirm the importance of oxidative stress in asthma, e.g., mice with reduced antioxidant defenses (nuclear factor erythroid 2–related factor 2 knockout mice)

ABBREVIATIONS: APAP, N-acetyl-para-aminophenol; BAL, bronchoalveolar lavage; ETS, environmental tobacco smoke; GSH, glutathione; HDM, house dust mite; IL, interleukin; PAS, periodic acid–Schiff; PCR, polymerase chain reaction; P450, cytochrome P450; 5-PP, 5-phenyl-1-pentyne; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction.
demonstrate enhanced susceptibility to allergic airway disease (Rangasamy et al., 2005; Williams et al., 2008). At sufficient doses APAP causes severe liver toxicity primarily through oxidative stress caused by its reactive metabolite, N-acetyl-p-benzoquinone-imine (McGill and Jaeschke, 2013). Hepatotoxic doses of APAP have been associated with extrahaepatic GSH loss and tissue injury in the nose and lung (Hart et al., 1995; Gu et al., 2005).

The effects of APAP at high doses on the liver and airways suggest an oxidative stress–based mechanism is plausible. Recent work in our laboratory focused on the potential for lower, nonhepatotoxic doses of APAP to cause oxidative stress in the airways. At near-therapeutic doses, APAP causes airway GSH loss and airway cellular oxidative stress response pathway induction, and potentiates acute airway responses to environmental tobacco smoke (ETS) (Smith et al., 2016). That APAP acts as a pro-oxidant in the respiratory tract suggests it could promote the development of asthma similar to other oxidant asthma risk factors such as ETS, ozone, and diesel exhaust (Gilmour et al., 2008). Our previous studies focused on the acute pro-oxidant effects of APAP on the airways. While they support a proposed mechanism for the APAP-asthma association, which may involve oxidative stress and the potentiation of the effects of ETS, they do not provide direct evidence linking APAP treatment with the development of future allergic disease. Thus, the overarching goal of the current study was to investigate the effects of APAP on allergic responses of the airways in a murine house dust mite (HDM) model of allergic airway disease. We hypothesized that APAP administration would enhance the development of allergic airway disease caused by HDM, similar to the enhancement of HDM-induced inflammation observed with other pro-oxidants such as ETS (Mitchell et al., 2012; Lanckacker et al., 2013).

Interestingly, our initial studies indicated that APAP did not enhance the response to HDM antigen, rather the opposite effect was observed. APAP effectively blocked the response to HDM. The approach of the experiments featured in this study then became to characterize and document the unexpected attenuation of the HDM response by APAP through measurements of airway inflammatory cell influx, cytokine mRNA profiles, HDM-specific serum immunoglobulin, and airway histologic presentation.

Materials and Methods

Experimental Approach. A HDM model of allergic airway disease was chosen for its environmental and human relevance since allergies to HDM are highly prevalent in humans, especially among those with asthma (Boulet et al., 1997; Calderon et al., 2015). The response to HDM in mice exhibits several of the principle features of allergic disease in humans, and importantly HDM produces a respiratory allergic response in mice that has been extensively characterized (Cates et al., 2004, 2007; Johnson et al., 2004; Maes et al., 2010). Immunologic tolerance in HDM models has been shown to occur after long-term exposure (Bracken et al., 2015). Therefore, our studies were limited to 2 weeks in duration and designed to examine changes in the early response to both minimal and maximal doses of HDM.

Mouse models of asthma have been defined as having two main phases, an early allergic sensitization phase, followed by a challenge phase (Maes et al., 2010). The timing of coexposures to other pro-oxidants such as ETS (e.g., during sensitization versus challenge) has been shown to produce pleiotropic disease outcomes in asthma models (Maes et al., 2010; Botelho et al., 2011; Lanckacker et al., 2013). Therefore, in our experiments investigating the effects of APAP on the response to HDM, APAP was given during either the first or second week of the HDM model exposure to account for the possibility of differential effects during earlier versus later phases of the model. A 100 mg/kg dose of APAP was used in this study based on dose-response relationships described in our previous work (Smith et al., 2016). This dose causes significant GSH loss throughout the respiratory tract at 1 hour, but importantly elicits peak blood APAP levels of 35 μg/ml, which are only slightly higher than therapeutic levels (5–20 μg/ml).

Mice and Dosing. Experiments were performed on 9–12-week-old female C57Bl/6J mice obtained from The Jackson Laboratory, Bar Harbor, ME. Mice were housed in American Association for Accreditation of Laboratory Animal Care accredited facilities at the University of Connecticut under standard environmental conditions (12-hour light-dark cycles at 23°C). Mice were housed over hardwood shavings (Sani-Chip Dry, P. J. Murphy Forest Products, Montville, NJ). Food (Laboratory Diet; PMI Nutrition International, St. Louis, MO) and tap water were provided ad libitum. All animal studies were reviewed and received prior approval by the University of Connecticut Institutional Animal Care and Use Committee (protocol A12-013).

All chemicals were obtained from Sigma Aldrich (St. Louis, MO), unless otherwise indicated, and were of USP grade or better. APAP (PubChem 1983, PubChem Compound Database, National Center for Biotechnology Information, https://pubchem.ncbi.nlm.nih.gov/compound/1983) dissolved in 37°C saline (10 mg/ml) was administered via i.p. injection. The cytochrome P450 (P450) inhibitor 5-phenyl-1-pentyne (5-PP), GFS Chemicals, Powell, OH) (PubChem 74573, PubChem Compound Database, National Center for Biotechnology Information, https://pubchem.ncbi.nlm.nih.gov/compound/74573) was given i.p. at a dose of 100 mg/kg (10 mg/ml in olive oil) 1 hour prior to APAP treatment (Morris, 2013). For euthanasia, mice were anesthetized by urethane injection (1.3 g/kg i.p.), followed by exsanguination through the abdominal aorta or cardiac puncture.

Murine Model of HDM Antigen-Induced Allergic Airway Disease. Based on the exposure protocol of Bracken et al. (2015), equal parts of Dermatophagoides pteronyssinus and Dermatophago- goides farinae lyophilized HDM extracts (Greer Laboratories, Lenoir, NC) were resuspended in phosphate-buffered saline at concentrations from 12.5 to 200 ng/dry weight/μl. The lyophilized HDM extract mixture contained 3131 endotoxin units/mg of HDM, and 3.32% D. pteronyssinus/ D. farina, and the same lot number was used throughout the experiments. The HDM suspension was delivered in the morning between 9:00 and 12:00 AM by intranasal instillation in a single 50 μl volume while the mice were lightly anesthetized with isoflurane (2.5%).

Bronchoalveolar Lavage (BAL) and Tissue Collection. Following euthanasia, lungs were lavaged in situ with phosphatebuffered saline (3 x 1 ml lavages). BAL fluid cells were pelleted at 2000g for 5 minutes, and lavage supernatant was collected and frozen at −20°C for total protein determination. Total protein was measured by the Lowry method (Lowry et al., 1951). Cell pellets were resuspended in phosphate-buffered saline containing 2% bovine serum albumin. Total cell counts were obtained with a hemocytometer and Turk’s solution. Slides of BAL cells were prepared by cytocentrifugation and stained with the Protocol Hema 3 system (Fisher Healthcare, Waltham, MA). Differential white blood cell counts were performed on a minimum of 200 cells. No eosinophils, neutrophils, or lymphocytes were observed in the vehicle control and APAP groups that received HDM vehicle instillations. Lungs collected for quantitative real-time polymerase chain reaction (qRT-PCR) analysis were inflated with and stored in aqueous RNA stabilization buffer containing saturating ammonium sulfate, 20 mM EDTA, and 25 mM sodium citrate at pH 5.2. When collected, blood was obtained via cardiac puncture from anesthetized mice and spun at 1000g for 10 minutes to obtain serum.

qRT-PCR. Total RNA was isolated from mouse lung tissue homogenates using an RNeasy kit from Qiagen (Hilden, Germany). A 1 μg dose of total RNA was used for first strand cDNA synthesis by an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time polymerase chain reaction (PCR) was performed using SYBR Green
as an indicator with a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). PCR reactions contained 10 ng of cDNA (4 μl), 500 nM of each primer (1 μl total), and 5 μl of 2X SYBR Green PCR Master Mix (10 μl total volume). The PCR was carried out according to the manufacturer’s recommended thermal cycling protocol. β-Actin was used as the internal reference control mRNA. Results are represented as the fold change in expression of target genes over control calculated using the 2−ΔΔCt method (Livak and Schmittgen, 2001). The genes selected for qRT-PCR analysis and the functional basis for their selection are provided in Table 1. Primer sequences (Table 2) were designed with the Life Technologies (Carlsbad, CA) OligoPerfect designer, or obtained from the Harvard Primer Bank (Harvard Medical School, Boston), and synthesized by Invitrogen (Life Technologies).

**Serum Immunoglobulin Measurement.** Detection of serum HDM-specific IgE and IgG was performed as described previously (Bracken et al., 2015). Briefly, Nunc MaxiSorp, flat-bottom plates (Thermo Scientific, Waltham, MA) were coated with 10 or 2 μg/ml of HDM extract in sodium bicarbonate buffer (pH 9.5) for IgE or IgG, respectively. Blocking was performed with BD OptiEIA assay diluent (BD Biosciences San Jose, CA). Serum samples were added for the IgE assay in 2-fold serial dilutions (1/20–1/2560) and for the IgG assay in 10-fold serial dilutions (1/20–1/200,000,000). For antibodies, biotin-SP-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL) and biotin-SP-conjugated goat anti-mouse IgE (Southern Biotech, Birmingham, AL) were coated with 10 or 2 μg/ml of HDM-specific IgE and IgG was performed as described previously (Smith et al., 2016), which indicated that at this dose and time point, APAP causes depletion of the antioxidant GSH throughout the respiratory tract and potentiates the effects of another oxidant, ETS. APAP was administered during either week 1 or 2 of the HDM model and mice were euthanized on day 12 (Fig. 1A). APAP was administered for no more than 5 days after the 2-day rest period in between weeks 1 and 2. On day 12 mice were euthanized, 24 hours following the last dose of HDM (Supplemental Fig. 1A). HDM produced a dose-dependent increase in the number of airway inflammatory cells as measured by the presence of increased eosinophils, neutrophils, and lymphocytes in the BAL fluid (Supplemental Fig. 1, B–D). The numbers of these cells in control mice groups were negligible, accounting for <0.5% of the cells present. Macrophage numbers were significantly higher than control at HDM doses of 5 μg or higher (Supplemental Fig. 1E). No consistent increase in BAL cellularity was observed with the 0.625 μg dose. The 1.25 μg dose was the lowest dose that caused a consistent, marked inflammatory response as indicated by statistically significant increases in eosinophils, neutrophils, and lymphocytes. Macrophages were increased to 1.4-fold of control at this dose, but the difference was not statistically significant. The response to 10 μg was no higher than that to 5 μg, suggesting these were maximally effective doses relative to inflammatory cell influx in this model.

**APAP Attenuates the HDM Inflammatory Response.** To examine the effect of APAP on airway inflammation induced by minimally (1.25 μg) and maximally (10 μg) effective doses of HDM, mice were treated daily with APAP or vehicle (control) 1 hour prior to HDM. APAP was administered at a non-hepatoxic dose of 100 mg/kg based on our previous studies (Smith et al., 2016), which indicated that at this dose and time point (1 hour) APAP causes depletion of the antioxidant GSH throughout the respiratory tract and potentiates the effects of another oxidant, ETS. APAP was administered during either week 1 or 2 of the HDM model and mice were euthanized on day 12 (Fig. 1A). APAP was administered for no more than 5 days since this is the maximum recommended duration of treatment of children (https://www.tYLENol.com/products/tYLENol-regular-strength-tablets#directions). As observed previously (see Supplemental Fig. 1), both the 1.25 and 10 μg doses of HDM resulted in significant influx of inflammatory cells in the BAL. Inflammatory cell levels in control and APAP groups were negligible. The eosinophilic, neutrophilic, and lymphocytic inflammatory cell response to the 1.25 μg dose of HDM was significantly attenuated in animals treated with APAP during week 2 (Fig. 1B). Macrophage levels averaged 1.6- to 2.8-fold over control in HDM (1.25 μg) and HDM-APAP (1.25 μg) groups but no statistical difference was detected among these groups. The attenuation of the HDM response was less pronounced at the 10 μg dose level, and was less consistent across

**Results**

**HDM Dose-Response Relationships.** Initial studies were aimed at defining the dose-response relationships for HDM to delineate both a minimally and maximally effective dose. Based on previous studies, five dose levels ranging from 0.625 to 10 μg were used (Bracken et al., 2015). Mice were administered HDM intranasally once daily for 2 weeks with a 2-day rest period in between weeks 1 and 2. On day 12 mice were euthanized, 24 hours following the last dose of HDM (Supplemental Fig. 1A). HDM produced a dose-dependent increase in the number of airway inflammatory cells as measured by the presence of increased eosinophils, neutrophils, and lymphocytes in the BAL fluid (Supplemental Fig. 1, B–D). The numbers of these cells in control mice groups were negligible, accounting for <0.5% of the cells present. Macrophage numbers were significantly higher than control at HDM doses of 5 μg or higher (Supplemental Fig. 1E). No consistent increase in BAL cellularity was observed with the 0.625 μg dose. The 1.25 μg dose was the lowest dose that caused a consistent, marked inflammatory response as indicated by statistically significant increases in eosinophils, neutrophils, and lymphocytes. Macrophages were increased to 1.4-fold of control at this dose, but the difference was not statistically significant. The response to 10 μg was no higher than that to 5 μg, suggesting these were maximally effective doses relative to inflammatory cell influx in this model.

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all cell types; however, significantly fewer eosinophils were observed in the APAP-treated animals at this HDM dose (Fig. 1B). For both HDM dose groups, APAP was more effective at attenuating the inflammatory response when given in week 2 than in week 1. No significant differences were observed between the control and APAP groups with respect to macrophage numbers (Fig. 1B). HDM at both doses significantly elevated BAL supernatant protein levels, a response blocked by APAP treatment during week 2 (Fig. 1C).

**Time Course of APAP’s Effect on HDM Response.** To determine the time course of the effects of APAP on the HDM response, we performed an experiment in which groups of mice treated with HDM or APAP and HDM (week 2) were euthanized on each day of the second week of the HDM model. The lungs of euthanized mice were lavaged and lung tissue samples were collected for determination of mRNA levels of inflammatory genes (Supplemental Fig. 2). Interestingly, the expression of the Th2 cytokines IL-4 and IL-5, the mucus forming Muc5AC gene, and the proinflammatory cytokine Mip2 (Fig. 3, A–D). The effects of APAP on gene expression were apparent on day 12 of the models. Specifically, the expression of IL-4 and Mip2 was significantly lower in groups treated with APAP. The IL-5 and Muc5AC responses were also attenuated by APAP; however, the effect did not attain statistical significance (P = 0.08 and 0.053, respectively). Notably, APAP was without effect on expression of any of these genes on day 10. The genes IL-13, CaSR, iNos, Nqo1 (Supplemental Fig. 2), and Cyp2E1 (Fig. 3E) were not significantly elevated over control levels by HDM and the degree of change was less than 1.5-fold in all cases. APAP itself did not alter the expression of these genes (Supplemental Fig. 2). Interestingly, the expression of Cyp2E1 was significantly lower than control in mice treated with HDM on day 12 of the model, a response prevented by APAP (Fig. 3E).

**Effect of P450 Inhibition.** To determine whether the attenuation of the HDM-induced inflammatory response was due to the parent APAP molecule or a P450 metabolite, we performed a separate experiment in which mice were treated with the P450 inhibitor, 5-PP; if a P450 metabolite was involved, it would be anticipated that the attenuating effects of APAP would be absent in the 5-PP-treated mice. 5-PP was administered 1 hour prior to APAP based on our previous studies in which it was shown that 5-PP at this dosage blocked the airway oxidant response to APAP (Morris, 2013; Smith et al., 2016). In the current study, 5-PP was administered for a maximum of 2 days (on days 8 and 9) to minimize the potential for toxicity and maintain effective P450 inhibition. Mice were euthanized on day 10 since this was the first day of the model in which the attenuating effects of APAP on inflammation were observed (see Fig. 2, A–C). The effect of APAP was apparent in the 5-PP-treated groups with respect to decreased eosinophils and neutrophils (Fig. 4, A and B). For neither cell type was the response different between the APAP- and 5-PP/ APAP-treated groups. 5-PP alone may have caused a decrease in inflammation (Fig. 4, A and B). However, this decrease was not statistically significant. In both the APAP- and 5-PP/ APAP-treated groups there were decreased lymphocytes, although no statistically significant differences were found.

### Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>β-Actin</td>
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<td>CCAAGAAAGGAAGCCTGGA</td>
</tr>
<tr>
<td>IL-4</td>
<td>CCAAGCGACTCTCTGTGGTGG</td>
<td>TGGGACATTCCTGTGTTCCAG</td>
</tr>
<tr>
<td>IL-5</td>
<td>CTCCTGTGCAACAGGAATGAGCG</td>
<td>TCTTCCATGTCTGTCAGCCCCCTGG</td>
</tr>
<tr>
<td>IL-13</td>
<td>CTCGGCCCTGCGTGGTCTGCCT</td>
<td>GTCTCTGTGTGTTGGTCGTCA</td>
</tr>
<tr>
<td>CaSR</td>
<td>AGCGAGGATGCTCTCCATGAGGT</td>
<td>ACTTTCTTGACAGACACATGAGGC</td>
</tr>
<tr>
<td>Muc5AC</td>
<td>CTGTGCAATATCCATCAAGGCCC</td>
<td>AAGGGGATAGCTGGCCTGGA</td>
</tr>
<tr>
<td>Mip2</td>
<td>CCAAGCCACGAGCTACAGG</td>
<td>GGTGGTCACACTCAAGGTGTCC</td>
</tr>
<tr>
<td>iNos</td>
<td>TGAAGAAGACCCCTTGTGCT</td>
<td>TTCTGCGCTGGCCACAGTGAG</td>
</tr>
<tr>
<td>Cyp2E1</td>
<td>GGGACATTCTCCTGTCTCCAGG</td>
<td>CTTAGGAAAACCCCTGCGGAC</td>
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<tr>
<td>Nqo1</td>
<td>TTTAGGGTCGTCTTGGCAA</td>
<td>GTCTCTCTGTAATGGGCGGAG</td>
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Macrophage levels were increased to approximately 2-fold of control by HDM and fewer macrophages were observed in the APAP-treated group, a response which was not different from the 5-PP/APAP group (Fig. 4D).

**Histologic Effects.** To confirm the results of previous endpoints, a qualitative histologic assessment of lungs from HDM-exposed mice (with or without APAP treatment) and controls was performed. No inflammation was apparent in vehicle- or APAP-treated control mice (Fig. 5, A and B). A focal and moderate degree of lung inflammation was present in HDM-exposed mice (Fig. 5C). Macrophages and polymorphonuclear cells were observed in perivascular and peribronchiolar...
clusters distributed throughout the majority of the lung lobes. Fewer clusters of inflammatory cells were observed in APAP-treated groups, and some lobes did not have any indication of inflammation (Fig. 5D). Both HDM and APAP + HDM groups had increased small airway PAS staining, indicative of enhanced mucus production (Fig. 5, G and H). The degree of PAS staining appeared to be somewhat reduced in the APAP + HDM groups (Fig. 5H). No evidence of increased PAS staining was observed in the control groups (Fig. 5, E and F).

**Effect of APAP on HDM-Specific Serum Immunoglobulin.**

HDM-specific IgE and IgG1 were not detectable in sera of mice on day 12 of the model in any treatment group (control, APAP, HDM, or HDM + APAP) (data not shown).

**Discussion**

The epidemiologic evidence suggesting APAP use may influence the pathogenesis of asthma is controversial and the plausibility of the model has not been tested in animal
models. Therefore, the objective of this study was to evaluate the effects of APAP in a murine HDM model of allergic airway disease. HDM produced a robust and dose-dependent increase in inflammation as measured by increased airway eosinophilia and neutrophilia, similar to that observed by other investigators using HDM models (Cates et al., 2004; Bracken et al., 2015). An approximately 2-fold increase in airway macrophages was consistently observed in our model; however, the change was not uniformly detected as statistically significant. Previous studies observed a maximal response at a 25 μg dose (Cates et al., 2004). The response to HDM in our model peaked at a 10 μg dose, likely influenced by a difference in the day on which BAL was performed [day 12 in our model, compared with day 15 in the Cates et al. (2004) study, and perhaps differences in HDM extract composition (lipopolysaccharide and protein levels)]. In preliminary studies using 1.25 and 10 μg doses of HDM, neutrophil and eosinophil numbers were lower on day 15 than day 12—signaling some resolution of inflammation over 2 days without exposure. Therefore, BAL was performed on day 12 for the majority of these studies to examine the progression of inflammation.

We hypothesized that APAP would enhance the response to HDM based on our previous studies, which indicated that APAP was an airway pro-oxidant (Smith et al., 2016). Unexpectedly, APAP caused a marked attenuation of the HDM response. A recently published report has shown that, in mice, maternal exposure to APAP during pregnancy and lactation did not enhance HDM-induced allergic airway disease in offspring (Lee et al., 2015). Our study is also consistent with a lack of causality between APAP and asthma as proposed by Heintze and Petersen (2013). Moreover, our results suggest APAP may actually have a therapeutic role relative to asthma pathogenesis.
In our model, APAP was more effective at blocking the HDM response at the 1.25 μg HDM dose level than at the 10 μg HDM dose level, suggesting that the attenuation might be overwhelmed at higher antigen burdens. APAP was more effective during week 2 of the HDM model despite the fact that mice had been previously exposed/sensitized to antigen for a week. This suggests that APAP has a greater effect on the exacerbation compared with the induction of allergic inflammation. The inflammatory endpoints we measured (in particular, airway eosinophilia and neutrophilia) are the result of differentially regulated processes (Lambrecht and Hammad, 2015). Therefore, the lack of an increase in any of these cell types or in BAL protein after APAP treatment is suggestive of a general anti-inflammatory effect. This is unexpected because APAP is thought to have weak anti-inflammatory effects (Graham et al., 2013). Furthermore, on day 12 APAP blocked the attenuating effect of HDM on Cyp2E1 expression, a gene that is not directly related to the HDM inflammatory response but is known to decrease in other allergic airway disease models (Stoilov et al., 2006).

Mild to moderate peribronchiolar and perivascular inflammation was observed in HDM-treated mice. Clusters of inflammatory cells were absent or less apparent in APAP-treated mice, confirming the decreased BAL inflammatory cell response of APAP-treated mice. The degree of inflammation observed in this study was less than that observed in our previous studies, which used a higher dose of HDM (Bracken et al., 2015). However, a similar perivascular and peribronchiolar clustering pattern was observed in both studies. Neither HDM-specific IgG1 nor IgE was detected in sera of HDM-treated mice. Bracken et al. (2015) reported low levels of these antibodies on day 15 of their model that used 25 μg intranasal instillation of HDM (5 days per week). The lack of IgG1 and IgE may be due to the shorter time period (12 versus 15 days) and lower HDM dosage (10 versus 25 μg) in the current study.

The gene expression analyses were intended to determine specific gene expression patterns caused by both the HDM and combination of HDM and APAP treatments. Il-4, Il-5, and Il-13 were chosen since they are primary effector cytokines of the Th2-type allergic response (Lambrecht and Hammad, 2015). Associated with both allergic and nonallergic inflammatory responses, Muc5AC, Mip2, and iNos are effectors of increased mucus glycoprotein production, neutrophil influx, and airway and vascular smooth muscle tone, respectively (Wolpe et al., 1989; Hamid et al., 1993; Coleman, 2002; Evans et al., 2015). Cyp2E1 is
ovalbumin and extracts of HDM, features of asthma in their mixed allergen (combination of treatment with CaSR antagonists blocked the characteristic expression were elevated in asthmatic humans and mice, and Yarova et al. (2015) observed that and inflammation in allergic asthma (Yarova et al., 2015). Other clear changes were observed except a decrease in mucin proteins such as induction relative to tissue collection or the influence of other histologic assessment, perhaps due to the timing of not translate to markedly different mucus in the qualitative day 12. The slight decrease in Muc5AC gene expression did not translate to markedly different mucus in the qualitative histologic assessment, perhaps due to the timing of Muc5AC induction relative to tissue collection or the influence of other mucin proteins such as Muc5b, which were not examined. No other clear changes were observed except a decrease in Cyp2E1 with HDM, which was reversed by APAP on day 12.

CaSR may be a gatekeeper of airway hyper-responsiveness and inflammation in allergic asthma (Yarova et al., 2015). Yarova et al. (2015) observed that CaSR mRNA and protein expression were elevated in asthmatic humans and mice, and treatment with CaSR antagonists blocked the characteristic features of asthma in their mixed allergen (combination of ovalbumin and extracts of HDM, Alternaria, and Aspergillus) model. We did not observe any significant changes in CaSR expression with HDM exposure in our studies. Additionally APAP attenuated the response to HDM without producing any alteration in CaSR expression. These results suggest that CaSR may not be the key to inflammation in all models of asthma, and may not be critical to HDM allergen sensitization.

The mechanism of APAP’s effect on the response to HDM is unknown; that 5-PP did not block the effect of APAP suggests the effect is not due to a P450 metabolite but more likely the parent compound or a (non-P450 metabolite). This explains the apparent discordance between the current results and our previous studies in which the acute pro-oxidant effects of APAP were blocked by 5-PP (Smith et al., 2016). It is possible that the repeat dosing paradigm in the current study has an antioxidant effect in the airways through upregulation of nuclear factor erythroid 2–related factor 2 (O’Connor et al., 2014). However, this seems unlikely because the nuclear factor erythroid 2–related factor 2–mediated gene Nqo1 was not induced throughout the time course. Our previous study demonstrated an acute pro-oxidant interaction between APAP and ETS. Perhaps rather than promoting asthma pathogenesis directly, APAP may predispose individuals to other pro-oxidant asthma causative factors such as ETS (Smith et al., 2016). The potential for APAP to potentiate the effects of other airborne oxidants such as ETS was not investigated here, and therefore remains an important consideration.

There were no differences in cytokine expression or BAL protein levels between the HDM and APAP + HDM groups on day 10, a time at which significant changes in BAL cellularity were observed. Thus, the changes in gene expression lagged the changes in cellularity, suggesting that the cytokine expression following APAP treatment is most likely an effect rather than the cause of the decreased inflammation by APAP. A possible mechanism is that APAP could be influencing the trafficking of immune cells. This concept is supported by an apparent differential effect of APAP on resident versus recruited cells. Specifically, APAP exerted profound effects on cells that are largely recruited to the lungs (eosinophils, neutrophils, and lymphocytes), whereas lesser effects were seen on resident cells. Macrophage numbers were decreased by APAP, but the effect was not as great as for eosinophils or neutrophils and did not consistently achieve statistical significance. Similarly, the effect of APAP on mucus cell metaplasia appeared to be of lesser magnitude. Decreased immune cell migration could be caused by activation of the cannabinoid receptor system by APAP, either by diverting metabolism of arachidonic acid to endocannabinoids through inhibition of prostaglandin synthesis or through a direct effect of its recently discovered metabolite and anandamide analog AM404 (Graham et al., 2013). Cannabinoid receptor activity has been shown to decrease the recruitment of immune cells (Lunn et al.,

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**Fig. 5.** HDM-exposed mice demonstrate moderate perivascular/peribronchiolar inflammation, which is largely absent in APAP-treated mice. Lungs were fixed in formalin, sectioned, and stained with hematoxylin and eosin (H&E) or PAS. (A–D) Arrows indicate examples of perivascular/peribronchiolar inflammation (H&E × 200). (E–H) Arrows indicate examples of mucus production by airway goblet cells (PAS × 200). Images are representative of lungs from each group indicated above each image column, and two independent experiments (n = 3 mice/group).
Activation of the cannabinoid system also enhances immune cell apoptosis in mice (Rieder et al., 2010). This effect may also be involved in the attenuation of the HDM model by APAP. However, inflammatory cell numbers were never increased in the APAP-treated group, suggesting that an effect on cell trafficking into the lungs is more likely than an enhanced apoptosis of cells within the lungs.

In conclusion, we provide direct biologic data that do not support the APAP hypothesis in asthma, a result in agreement with recent epidemiologic and animal studies. It is possible the association between APAP use and asthma can be explained by confounding factors. Importantly, our results indicate that HDM-induced allergic airway disease is markedly attenuated by APAP, indicating the possibility of a novel therapeutic effect of APAP. More studies will be needed to confirm these results in other models of airway inflammation and asthma and to determine the mechanism of the observed effects. The mechanistic insights provided by the modulation of allergic airway disease by APAP may provide novel information on the pathogenesis of asthma and potential novel therapeutic targets.

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Authorship Contributions

Participated in research design: Smith, Thrall, Cloutier, Manautou, Morris.

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