



Published in final edited form as:

Exp Lung Res. 2013 November ; 39(9): 365–378. doi:10.3109/01902148.2013.829537.

High-fat diet promotes lung fibrosis and attenuates airway eosinophilia after exposure to cockroach allergen in mice

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Abstract

Obesity is an important risk factor for asthma but the mechanistic basis for this association is not well understood. In the current study, the impact of obesity on lung inflammatory responses after allergen exposure was investigated. C57BL/6 mice maintained on a high-fat diet (HFD) or a normal diet (ND) after weaning were sensitized and challenged with cockroach allergen (CRA). Airway inflammation was assessed based on inflammatory cell recruitment, measurement of lung Th1-Th2 cytokines, chemokines, eicosanoids, and other proinflammatory mediators as well as airway hyperresponsiveness (AHR). CRA-challenged mice fed a HFD exhibited significantly decreased allergen-induced airway eosinophilia along with reduced lung IL-5, IL-13, LTC₄, CCL11, and CCL2 levels as well as reduced mucus secretion and smooth muscle mass compared to ND fed mice. However, allergen-challenged HFD fed mice demonstrated significantly increased PAI-1 and reduced PGE₂ levels in the lung relative to corresponding ND fed mice. Interestingly, saline-exposed HFD fed mice demonstrated elevated baseline levels of TGF- α 1, arginase-1, hypoxia-inducible factor-1 α , and lung collagen expression associated with decreased lung function compared to corresponding ND fed mice. These studies indicate that a HFD inhibits airway eosinophilia while altering levels of PAI-1 and PGE₂ in response to CRA in mice. Further, a HFD can lead to the development of lung fibrosis even in the absence of allergen exposure which could be due to innate elevated levels of specific profibrotic factors, potentially affecting lung function during asthma.

Keywords

allergic inflammation; fibrogenic factors; obesity; reduced eosinophilia; Th2 cytokines

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

INTRODUCTION

Studies in humans and in mouse models have established that obesity is an important risk factor for asthma [1] and is associated with increased severity as well as poor asthma control relative to non-obese asthmatics despite similar symptoms perception scores in humans [2, 3]. Obesity has been shown to be associated with an increased risk for development of airway hyperresponsiveness (AHR) in humans [4]. Further, mice with obesity due to a genetic deficiency or exposure to a high-fat diet (HFD) demonstrate innate AHR to methacholine [5, 6]. In further support, weight loss in severely obese patients with asthma was shown to improve respiratory symptoms and lung function [7, 8]. Taken together, these findings suggest that asthma in the obese is associated with impaired lung function.

While the connection between obesity and the incidence of asthma is well established, the mechanistic basis for this is still not entirely clear. Further, the effect of obesity on inflammatory cell recruitment remains conflicted. For instance, similar to obese patients with difficult-to-treat asthma where body mass index was inversely related to sputum eosinophils [9], studies in mice that are obese either due to a genetic deficiency [5] or a HFD [10] have demonstrated decreased recruitment of inflammatory cells, especially eosinophils, to the airways after allergen (ovalbumin [OVA]) exposure compared to normal mice. In contrast, other studies have shown that OVA-challenged HFD fed obese mice or genetically modified (leptin-deficient *ob/ob*) mice exhibit enhanced eosinophilopoiesis and eosinophil recruitment from bone marrow (BM) to lung tissues with delayed transit into the airway lumen and longer presence in lung peribronchiolar segments due to overproduction of Th1/Th2 cytokines and chemokines [11, 12]. In support of this, treatment of human eosinophils with physiological concentrations of leptin, an adipokine that is elevated during obesity, increased their migration toward eotaxin *in vitro* [13]. More recently, studies in mice have shown that diet-induced obesity (DIO) results in increased airway eosinophilia and enhanced lung remodeling resulting in higher AHR in experimental chronic allergic asthma [14]. Additionally, mice deficient in adiponectin, an anti-inflammatory adipokine that increases insulin sensitivity and is reduced during obese conditions, demonstrated increased accumulation of eosinophils in the airways after OVA challenge compared with wild-type (WT) mice, although it is important to note that these mice were not obese [15].

Asthma in the humans who are obese is recognized as a unique phenotype that is associated with AHR and lack of eosinophilic inflammation [16, 17], although the underlying mechanistic basis for this remains poorly understood. In the present study, we have evaluated the development of allergic airway inflammation in the setting of obesity in mice maintained on a HFD in terms of inflammatory cell recruitment, production of inflammatory mediators, profibrogenic factors and AHR after exposure to cockroach allergen (CRA), an allergen known to contribute to increased asthma morbidity, especially in inner-city lower socioeconomic groups [18] among whom obesity is also a major concern [19].

MATERIALS AND METHODS

Mouse Strain and Diet

C57BL/6J male mice bred in-house were maintained on a HFD with 60% kcalories from fat (F3282, Bio-Serv, Frenchtown, NJ) after weaning at 3 weeks. Another group of age and gender matched C57BL/6J mice were fed the standard normal diet (ND) with 18% kcalories from fat (Harlan Laboratories, Madison, WI). Both groups of mice fed ad libitum. All studies involving mice were performed following standards and procedures approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Induction of allergic airway inflammation—After 12 weeks, mice were sensitized by two routes (i.p. and s.c.) with 10 μ g of CRA (Hollister Stier, Spokane, WA) and 1 mg of aluminum hydroxide in saline (day 0). One week later (day 7), mice received a second injection (s.c.) of 1 μ g CRA containing 0.5 mg of aluminum hydroxide in saline. On day 14 and 21, mice were challenged (i.n.) with 1 and 4 μ g of CRA, respectively. Control mice were sensitized with aluminum hydroxide in saline and were challenged with saline instead of CRA. Mice were maintained on their respective diets throughout the study. After 24 hours of the last challenge, mice were weighed and assessed for the development of allergic airway inflammation.

Measurement of airway responsiveness—According to the previously published protocols [20, 21], pulmonary function in saline and allergen-exposed mice fed a HFD or ND was assessed by invasive plethysmography (FinePointe™ RC system, Buxco, Wilmington, NC). Changes in pulmonary resistance (R_L) and dynamic compliance (C_{dyn}) in response to saline followed by increasing concentrations of inhaled methacholine (3–50 mg/mL) were continuously monitored. R_L and C_{dyn} values at 50 mg/mL of inhaled methacholine are shown.

Blood, bronchoalveolar lavage fluid (BALF), and lung tissue collection—Blood was collected in the presence of buffered citrate from mice (non-fasting) by cardiac puncture after euthanasia. Samples were centrifuged and the plasma collected was stored at -80°C for subsequent assays. Lungs were lavaged with 1 mL of saline. Total and differential cell counts in the BALF were determined based on morphological criteria from cytospin slides stained with Hema 3 staining system (Fisher Diagnostics, Middletown, VA). For further evaluation, BALF supernatant was stored at -80°C . Right lungs were snap-frozen and stored at -80°C while left lungs were perfused with 4% paraformaldehyde to preserve pulmonary structure, fixed in 4% paraformaldehyde for 48 hours at 4°C and paraffin-embedded.

Measurement of total cholesterol and triglycerides—Plasma triglyceride and total cholesterol levels were measured by the Clinical Pathology Laboratory at the College of Veterinary Medicine, University of Minnesota, using a Beckman AU480 chemistry analyzer (Beckman Coulter, Inc., Brea, CA).

Measurement of CCL11, CCL2, and TGF- β 1—According to the manufacturers' recommendations, levels of CCL11 (eotaxin-1), CCL2 (MCP-1), and TGF- β 1 in BALF were measured using ELISA kits (R&D Systems, Minneapolis, MN). Optical density of the color

developed was measured at 450/570 nm with a microplate reader (FLUOstar OPTIMA, BMG LABTECH, Durham, NC) and the concentration of each chemokine in the BALF was determined against a standard curve generated. Detection limit for each cytokine was 15.6 pg/mL

Measurement of lung Th1/Th2 cytokines—Protein concentration in supernatants of lung tissue homogenates prepared in lysis buffer (PBS containing 1% Triton X-100, 2 mM PMSF, and protease inhibitors) was measured (BCA Protein Assay Kit, Pierce Biotechnology, Rockford, IL). Th1 (IL-2 and IFN- γ)/Th2 (IL-4, IL-5, IL-13) cytokine and TNF- α levels in the supernatants were determined using Flex Set kits (BD Biosciences, San Diego, CA), according to the manufacturer with a FACScan flow cytometer equipped with CellQuest Pro™ Software (BD Biosciences) for data acquisition and FlowJo Software (Tree Star, Inc., Ashland, OR) for analysis or a FACSCanto II flow cytometer (for IL-13) with FACSDiva™ Software for data acquisition and analysis. Level of each cytokine was expressed as pg cytokine/mg protein.

Lung Histology

Paraffin-embedded tissue sections (4 μ m thick) were stained with Harris Modified Hematoxylin and Shandon Instant Eosin (Thermo Fisher Scientific Co., Pittsburgh, PA) to determine cellular infiltration. Stained slides were examined using a Nikon Microphot EPI-FL microscope and images were captured with an Olympus DP71 camera. Analysis for infiltrated eosinophils was performed by immunohistochemical staining of sections for eosinophil-specific major basic protein (MBP) with rat mAb against murine MBP as described in our previous studies [22]. Positively stained cells were counted and expressed as the average number of MBP-positive cells/microscopic field (magnification of \times 400). Expression of α -smooth muscle actin (SMA) was evaluated using mAb against murine SMA and the area of the peribronchial smooth muscle layer was quantitated by ImageJ image analysis system as described earlier [23]. Results were expressed as SMA-positive area (μ m²) per mm basement membrane length (BML). Arginase-1 expression was evaluated using goat polyclonal antibodies against human arginase-1 (2 μ g/mL, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) [24] and quantitated by ImageJ. Results were expressed as arginase-1 positive area (μ m²) per 100 μ m BML. Expression of TGF- β 1 was detected with rabbit polyclonal antibodies against TGF- β 1 [23]. Rat IgG (for MBP), mouse IgG (for SMA), rabbit IgG (for TGF- β 1), or goat IgG (for arginase-1) were used as control antibodies. Airway mucus production assessed by staining lung sections with periodic acid-Schiff's (PAS) reagent (Sigma-Aldrich®, St. Louis, MO) was quantitated by ImageJ and expressed as PAS-positive area (μ m²) per 100 μ m BML. Masson's trichrome stain (Sigma-Aldrich®) was used to assess lung collagen deposition. A total of 4–6 images of randomly selected non-overlapping fields were captured per lung section at \times 40 magnification and the area of trichrome-positive staining in the captured images was measured using Fiji, an image processing package based on ImageJ [25]. Results are expressed as trichrome positive area (μ m²) per field.

Western blot analysis—Lung tissue was homogenized in radioimmunoprecipitation assay (RIPA) buffer and total protein in the supernatants was measured (BCA Protein Assay

Kit, Thermo Fisher Scientific Co.). Expression of PAI-1, TGF- β 1, and hypoxia-inducible factor (HIF)-1 α in lung lysates was evaluated using 20 (for HIF-1 α), 40 (for PAI-1), or 80 (for TGF- β 1) μ g protein per lane. Rabbit polyclonal antibodies against PAI-1 (0.4 μ g/mL), TGF- β 1 (1 μ g/mL), or HIF-1 α (0.2 μ g/mL, all from Santa Cruz Biotechnology, Inc.) followed by HRP-conjugated goat anti-rabbit IgG (1:5000, Cell Signaling Technology, Danvers, MA) were used for detection of protein bands. HRP-conjugated anti-mouse β -actin (1:5000, Santa Cruz Biotechnology, Inc.) was used for monitoring the levels of β -actin expression in lung tissue as an internal control. Bound antibodies were detected using Immobilon Western Chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA) and bands were visualized on X-ray films, which were then scanned and analyzed using ImageJ. Density of PAI-1, TGF- β 1 (25 kD mature/active form), and HIF-1 α bands was normalized against β -actin after background subtraction in each case.

Evaluation of eicosanoids—Lung tissue eicosanoids were assessed as described previously [26]. Homogenized lung tissue (30–60 mg) was spiked with 1 pmol each of deuterated internal standards (15S-HETE-d8, PGE₂, and LTC₄-d5), vortexed and centrifuged at 4000 g for 10 minutes at 4°C. The aqueous phase was loaded onto Strata-X solid phase extraction columns (Phenomenex, Torrance, CA) conditioned with methanol and equilibrated with water. Proteins and other polar molecules were removed by washing columns with water and eicosanoids were eluted with methanol. The eluate was freeze-dried, resuspended in methanol and analyzed by Liquid Chromatography-Mass Spectrometry (LC-MS/MS) analysis (Shimadzu Prominence 2D HPLC coupled to an AB/Sciex API 4000 QTrap MS spectrometer) [27]. Chromatographic separations were achieved using a 2.1 \times 100 mm Agilent Zorbax Eclipse C18 column with a 3.5 micron particle size by gradient elution with solvent A (70:30:0.1 water:acetonitrile:acetic acid) and solvent B (50:50 acetonitrile:isopropanol) at a flow rate of 400 μ L/minute (0–1 minutes 0% solvent B, 10–15 minutes 100% B, 16–23 minutes 0%B). Mass spectrometry data were collected using multiple reaction monitoring in negative mode. Desolvation and initial ion focusing was achieved using electrospray ionization with ionspray voltage of –4500 V, declustering potential of –70 V and entrance potential of –10 V. Eicosanoids of interest were fragmented using collision-induced dissociation with collision energy of –30 V. All analytes were quantified using stable isotope dilution. Level of eicosanoid was expressed as ng/g lung tissue.

Statistical Analysis

Results are expressed as mean \pm SEM. Statistical significance was determined using the unpaired Student's *t*-test. A *P* value less than 0.05 was considered as significant. To adjust for multiple comparisons, a Bonferroni correction was applied and a *P* value less than 0.0125 (0.05/4) was used to assess statistical significance. However, to highlight any differences that might be nonsignificant after applying a Bonferroni correction but significant by Student's *t*-test, *P* value determined by Student's *t*-test and the Bonferroni adjusted *P* value are provided in the section “Results”.

RESULTS

Mice Fed a HFD Exhibit Decreased Cellular Inflammation After Allergen Challenge

Mice maintained on a HFD for 9 weeks after weaning were significantly heavier than mice on a ND at the time of exposure to CRA (38.96 ± 0.927 [HFD] versus 29.13 ± 0.9 [ND], $P < .001$, Bonferroni adjusted $P < .0125$). After allergen challenge (12 weeks on diet), HFD fed mice exhibited a 30% increase in body weight compared to ND fed mice and exposure to CRA did not have any effect on body weight in either group (Figure 1A). Previous studies have shown that C57Bl/6 mice maintained on a HFD for 9–12 weeks as in the present study demonstrate weight gain, increased adiposity and fasting blood glucose levels along with impaired glucose tolerance and insulin responsiveness [28]. In the current study, associated with the increased body weight, HFD fed mice had higher non-fasting plasma total cholesterol levels relative to ND mice which remained unaltered after CRA challenge (121.2 ± 11.3 [HFD saline] and 81.25 ± 11.4 [ND saline] versus 127 ± 10.8 [HFD CRA] and 88.0 ± 8.9 [ND CRA], $P < .01$, Bonferroni adjusted $P < .0125$). Plasma triglyceride levels, however, were similar in all four groups of mice (124.1 ± 16.5 [HFD saline] and 102.7 ± 6.9 [HFD CRA] versus 117.2 ± 27.5 [HFD saline] and 100.4 ± 15.7 [HFD CRA]). These values appear to be consistent with previous reports where mice were fed a HFD either identical to the one used in our study [29, 30] or a comparable HFD [11]. In ND fed mice, a prominent increase in cellular inflammation was evident in the airways after CRA challenge, as indicated by an increased number of inflammatory cells in the BALF (Figure 1, B, $\#P < .001$, Bonferroni adjusted $P < .0125$) and peribronchial lung tissue (Figure 1C, [H&E stain]). In contrast, in the CRA-challenged HFD fed mice, although cellular recruitment to the airways was higher than in the corresponding saline-exposed group, it was significantly lower than in CRA-challenged ND fed mice (Figure 1, B, $**P < .001$, Bonferroni adjusted $P < .0125$).

Differential Leukocyte Recruitment to the Lungs of ND and HFD Fed CRA-Challenged Mice and AHR

Differential cell counts indicated that the number of eosinophils, neutrophils, and lymphocytes, but not macrophages, in the BALF of CRA-challenged ND mice was significantly higher than in saline-exposed mice (Figure 2A, left and right panels, $\#P < .005$, Bonferroni adjusted $P < .0125$). CRA-challenged HFD mice on the other hand, exhibited an increase only in the number of BALF neutrophils ($\#P < .04$, Bonferroni adjusted $P > .0125$) and macrophages ($\#P < .001$, Bonferroni adjusted $P < .0125$) compared to corresponding control mice (Figure 2A, right panel). Importantly, the number of eosinophils, neutrophils, and lymphocytes were significantly lower in CRA-challenged HFD fed mice than in the ND fed counterparts ($**P < .002$, Bonferroni adjusted $P < .0125$). No significant difference was observed in the number of BALF macrophages between the CRA-challenged groups (Figure 2A, right panel). Lung tissue MBP-positive eosinophils were also markedly higher in allergen-challenged ND fed mice compared to saline-exposed mice (Figure 2B and C, $\#P < .001$, Bonferroni adjusted $P < .012$). While the number of eosinophils in the lungs of allergen-challenged HFD mice was also higher than in corresponding control mice ($\#P < .03$, Bonferroni adjusted $P > .0125$), it was significantly lower relative to allergen-challenged ND fed mice ($**P < .002$, Bonferroni adjusted $P < .0125$).

Consistent with increased recruitment of inflammatory cells, CRA-challenged ND fed mice exhibited increased AHR as demonstrated by the elevated R_L in response to inhaled methacholine at 50 mg/mL relative to corresponding saline-exposed mice by invasive plethysmography (Figure 2D, upper panel, $^{\#}P < .001$, Bonferroni adjusted $P < .0125$ for ND CRA versus ND saline). Surprisingly, despite the reduced cellular inflammation and eosinophilia, CRA-challenged HFD fed mice also exhibited increased R_L relative to corresponding saline-exposed mice ($^{\#}P = .009$, Bonferroni adjusted $P < 0.125$ for HFD CRA versus HFD saline) similar to ND fed mice. More interestingly, saline-exposed HFD fed mice demonstrated inherently higher R_L compared to saline-exposed ND fed mice at 50 mg/mL inhaled methacholine ($*P = .006$, Bonferroni adjusted $P < .0125$ for HFD saline versus ND saline). Further, while CRA-challenged ND fed mice exhibited reduced C_{dyn} compared to saline-exposed mice (Figure 2D, lower panel, $^{\#}P < .001$, Bonferroni adjusted $P < .0125$ for ND CRA versus ND saline), C_{dyn} was found to be inherently low in saline-exposed HFD fed mice compared to saline-exposed ND fed mice ($*P = .002$, Bonferroni adjusted $P < .0125$) and was similar to C_{dyn} in CRA-challenged HFD fed mice. These results suggest that HFD fed mice may be predisposed to AHR independent of allergen-induced airway inflammation.

Allergen-Challenged HFD Fed Mice Exhibit Reduced Lung Cytokine, Chemokine, and Eicosanoid Levels

Since allergic airway inflammation is mediated by a specific cytokine milieu, we assessed levels of the major Th1/Th2 cytokines and the proinflammatory cytokine TNF- α in lung tissue (Table 1). Maintenance on a HFD by itself did not appear to alter the levels of Th1/Th2 cytokines relative to a ND since the levels of these cytokines were similar in HFD and ND fed mice after saline exposure. CRA challenge did not significantly alter lung IFN- γ , IL-2, or IL-4 levels in either group relative to their respective control groups. However, in allergen-challenged HFD fed mice, IL-5 and IL-13 expression in lung tissue was found to be lower compared to allergen-challenged ND fed mice (Table 1, $**P < .03$ and $< .02$, respectively, Bonferroni adjusted $P > .0125$ for both) as was that of TNF- α ($**P = .008$, Bonferroni adjusted $P < .0125$). In addition, level of CCL11 and CCL2 was significantly suppressed in allergen-challenged HFD fed mice compared to corresponding ND fed mice (Table 1, $**P < .001$, Bonferroni adjusted $P < .0125$), correlating with the relative reduction in lung tissue eosinophils (Figure 2) and macrophages (data not shown) in these mice.

Since leukotrienes and eicosanoids are known mediators of inflammatory responses in allergic asthma [31], LTC $_4$ and PGE $_2$ in the lungs of CRA-challenged HFD and ND fed mice was measured (Table 1). CRA challenge increased LTC $_4$ levels in ND fed mice relative to saline controls ($^{\#}P < .04$, Bonferroni adjusted $P > .0125$) but failed to induce LTC $_4$ expression in HFD fed mice, with LTC $_4$ levels in the latter group being lower than in CRA-challenged ND fed mice ($**P < .04$, Bonferroni adjusted $P > .0125$ for ND CRA versus HFD CRA). Further, while CRA-challenge had no effect on lung PGE $_2$ levels in ND fed mice, levels of this eicosanoid in CRA-challenged HFD fed mice were lower compared to the corresponding saline-exposed group ($^{\#}P < .03$, Bonferroni adjusted $P > .0125$) as well as CRA-challenged ND fed mice ($**P < .02$, Bonferroni adjusted $P > .0125$).

Increased Expression of TGF- β 1 in Airways of HFD Fed Mice

In addition to the various cytokines and chemokines, we evaluated expression of TGF- β 1 in the lungs of allergen-challenged ND and HFD fed mice since TGF- β 1 plays an important role in the regulation of allergen-induced airway inflammation and remodeling [32]. TGF- β 1 levels in BALF of CRA-challenged ND fed mice were significantly higher compared to corresponding control mice (Figure 3A, $^{\#}P < .012$, Bonferroni adjusted $P < .0125$). Surprisingly, TGF- β 1 levels in saline-exposed HFD fed mice were elevated and higher than in saline-exposed ND fed mice ($*P < .025$, Bonferroni adjusted $P > .0125$). More importantly, in spite of the reduced cellular inflammation in CRA-challenged HFD fed mice, TGF- β 1 levels were as high as in CRA-challenged ND mice. Lung tissue TGF- β 1 levels in HFD fed mice by Western blot analysis indicated similar trends (Figure 3B, $*P < .025$, Bonferroni adjusted $P > .0125$ for HFD saline versus ND saline). Further, immunohistology of lung tissue from CRA-challenged ND fed mice revealed that inflammatory cells recruited to the airways are a major source for TGF- β 1 followed by airway epithelial and endothelial cells (Figure 3C). In HFD fed mice with decreased cellular inflammation, airway epithelial cells appear to be the major cell type associated with TGF- β 1 expression followed by endothelial cells. More intense staining was observed in the airway epithelium of saline-exposed and CRA-challenged HFD fed mice in comparison to the airway epithelium of ND fed counterparts. Overall, TGF- β 1 levels in the lungs of HFD fed mice are inherently elevated.

TGF- β 1 is known to induce collagen expression and promote subepithelial fibrosis resulting in tissue remodeling in asthmatic airways [33]. Since levels of this profibrogenic mediator appear to be inherently elevated in the lungs of HFD fed mice, we investigated whether HFD fed mice exhibit inherent fibrosis by evaluating total lung collagen deposition using Masson's trichrome stain (Figure 3D). No difference was observed in the trichrome-positive area between the CRA-challenged and saline-exposed groups in ND fed mice. This is not surprising since increased collagen deposition is largely observed in remodeled lungs in response to chronic and not acute allergen exposure. Interestingly, trichrome-positive area in saline-exposed and CRA-challenged HFD fed mice tended to be greater than in corresponding ND fed counterparts ($^{**}P < .04$, Bonferroni adjusted $P > .0125$ for ND CRA versus HFD CRA). These findings are suggestive of increased collagen deposition in the lungs of HFD fed mice regardless of allergen exposure.

Expression of PAI-1, HIF-1 α , and Arginase-1 in Lungs of HFD Fed Mice

Expression of PAI-1, which is elevated during obesity [34] and is known to contribute to the pathogenesis of asthma by promoting extracellular matrix deposition and lung remodeling [35], was evaluated. PAI-1 levels in lung tissue of saline-exposed ND and HFD fed mice were not significantly different nor did CRA-challenge induce PAI-1 expression in ND fed mice (Figure 4A), however, PAI-1 levels in CRA-challenged HFD fed mice were significantly higher not only compared to corresponding saline-exposed mice ($^{\#}P < .012$, Bonferroni adjusted $P < .0125$) but also CRA-challenged ND fed mice ($^{**}P < .012$, Bonferroni adjusted $P < .0125$). Next, we examined expression of HIF-1 α , given that it plays a role in promoting allergic airway inflammation [36] and in inducing fibrosis [37]. CRA challenge markedly induced HIF-1 α expression in the lungs of ND fed mice compared

to saline-exposed mice consistent with increased allergen-induced airway inflammation (Figure 4B, $^{\#}P < .012$, Bonferroni adjusted $P < .0125$). Interestingly, in HFD fed mice, HIF-1 α expression in the saline-exposed group was higher than in saline-exposed ND fed mice ($^*P < .05$, Bonferroni adjusted $P > .0125$) and similar to levels in the corresponding CRA-challenged group. Expression of arginase-1, another molecule known to be increased in asthma and contribute to airway remodeling [38] was examined by immunohistology. CRA challenge induced arginase-1 expression in ND fed mice by both airway epithelial cells as well as inflammatory cells (Figure 4C, left panels). In HFD fed mice, arginase-1 expression was largely noted in the airway epithelium (Figure 4C, right panels). Since CRA-challenged HFD fed mice exhibit reduced recruitment of inflammatory cells in the lung tissue compared to ND fed mice, arginase-1 expression in the airway epithelium of HFD and ND fed mice was quantitated (Figure 4D). Airway epithelial arginase-1 expression in saline-exposed HFD fed mice was significantly higher than in ND fed counterparts ($^*P < .012$, Bonferroni adjusted $P < .0125$). In contrast to ND fed mice where CRA induced arginase-1 expression ($^{\#}P < .012$, Bonferroni adjusted $P < .0125$ for ND saline versus ND CRA), CRA challenge did not further induce airway epithelial arginase-1 expression in a significant manner in the HFD fed group. These data suggest that basal arginase-1 levels are elevated in HFD fed mice.

Mucus Secretion and Smooth Muscle Mass are Reduced in Airways of Allergen-Challenged HFD Fed Mice

Mucus hypersecretion, which can lead to plugging and airflow limitation during allergic airway inflammation [39] was examined (Figure 5, A). In contrast to CRA-exposed ND fed mice that demonstrate increased mucus production relative to saline-exposed mice ($^{\#}P < .01$, Bonferroni adjusted $P < .0125$), no mucus secretion was detected in CRA-challenged HFD fed mice ($^{**}P < .01$, Bonferroni adjusted $P < .0125$ for ND CRA versus HFD CRA) and was similar to that observed in corresponding saline-exposed mice. We also examined airway smooth muscle (ASM) mass in these mice since ASM hyperplasia and hypertrophy is observed during allergen-induced airway inflammation [40]. Immunohistology revealed that although ASM mass in CRA-challenged HFD fed mice was higher than in corresponding control mice (Figure 5, B, $^{\#}P < .05$, Bonferroni adjusted $P > .0125$), it was lower than in CRA-challenged ND fed mice ($^{**}P < .02$, Bonferroni adjusted $P > .0125$).

DISCUSSION

While several studies have established a connection between obesity and asthma, the mechanistic basis is unclear and questions as to how obesity or increased weight gain might cause or worsen asthma remain unanswered. However, one consistent finding is that relative to non-obese conditions, asthma in the obese is associated with increased AHR and worsening lung function both in humans [1, 4, 16, 41] and mice [5, 6]. In the current study, we evaluated the impact of increased weight gain on the development of airway inflammation in response to CRA, a common inner city allergen, in mice maintained on a HFD for 12 weeks.

CRA challenge resulted in the development of airway eosinophilia (BALF and lung tissue) associated with elevated levels of lung Th2 cytokines (IL-5, IL-13), TNF- α , CCL11, CCL2, and LTC₄ as well as mucus hypersecretion and smooth muscle hyperplasia in ND fed mice. However, in comparison to ND fed counterparts, airway eosinophilia in the CRA-challenged HFD fed mice was markedly attenuated as were levels of IL-5, IL-13, TNF- α , CCL11, CCL2, and LTC₄ as well as mucus secretion and smooth muscle hypertrophy. Reduced airway eosinophilia in CRA-challenged HFD fed mice relative to corresponding ND mice may be due to decreased trafficking and recruitment from the BM. This is supported by the finding that IL-5, CCL11, and LTC₄, which are important for eosinophil release from the BM [42] and recruitment to inflammatory sites [43, 44], are present at significantly lower levels in CRA-challenged HFD fed mice than in ND fed mice. A previous study in HFD fed mice challenged with OVA also demonstrated decreased lung eosinophilia and IL-5 [10], even though another study in DIO mice with the same allergen demonstrated increased lung tissue eosinophils, BALF IL-5, eotaxin-1, and TNF- α levels [11]. However, even in this latter study, eosinophils in the BALF were significantly reduced in OVA-challenged HFD fed mice relative to ND fed mice similar to the previous finding as well as ours. Exposure of DIO mice to chronic OVA challenge on the other hand has been shown to result in increased eosinophil recruitment [14]. On the whole, parameters such as duration on HFD (12 weeks [current study], 8 weeks [10], or 10 weeks [11]) and consequently the variations in increase in body weight of the animals as well as the type and concentration of allergen (CRA versus OVA) and duration of exposure (acute versus chronic) are all likely to affect the outcome in terms of systemic and lung inflammatory responses, thus accounting for these varying observations.

Despite the reduced airway eosinophilia and attenuated Th2 cytokine response, CRA-challenged HFD fed mice demonstrated increased R_L and decreased C_{dyn} in response to methacholine challenge similar to CRA-challenged ND fed mice. This is somewhat similar to the asthma phenotype described in obese humans where there is AHR with lack of eosinophilic inflammation [16, 17]. Surprisingly, even saline-exposed HFD fed mice demonstrated significantly decreased C_{dyn} relative to corresponding ND fed mice suggesting that obesity or increased weight gain by itself may play a role in reducing pulmonary volume and/or elasticity even in the absence of allergen exposure. Previous studies suggest that abnormal fibrosis associated with increased collagen occurs in adipose tissue during the development of obesity [45]. We found that lung collagen expression in saline-exposed HFD fed mice tended to be higher than in corresponding ND fed mice and correlated with expression of TGF- β 1, a known inducer of collagen expression [46], which was higher in BALF and lung tissue of saline-exposed HFD fed mice relative to corresponding ND fed mice. Although inflammatory cells such as eosinophils and macrophages constitute a significant source of TGF- β 1 in allergic airways [33], in the absence of a strong influx of inflammatory cells in HFD fed CRA-challenged mice, airway epithelial cells appear to be a significant source for TGF- β 1 in these mice. Further, TGF- β 1 expression is known to be elevated in adipose tissue [47].

Recent studies have shown that asthmatic patients with increased body weight and diminished lung function have elevated PAI-1 levels, suggesting that the negative

association between obesity and poor lung function may in part be mediated by PAI-1 [48]. PAI-1 plays a role in increasing collagen deposition during allergic asthma [35] and promoting AHR as well as airway remodeling [49]. While CRA challenge resulted only in a modest increase in PAI-1 expression in comparison to the saline-exposed group in the lungs of ND fed mice, PAI-1 levels in CRA-challenged HFD fed mice were significantly higher relative to corresponding saline-exposed mice as well as allergen-challenged ND fed mice. The striking increase in PAI-1 levels in this group may be a combined effect of allergen exposure as well as that of leptin, which is elevated during obesity and is known to upregulate PAI-1 expression [50]. Additionally, like TGF- β 1, airway epithelial cells in saline-exposed HFD fed mice expressed significantly higher levels of arginase-1 than corresponding ND fed mice. Arginase is an enzyme that converts L-arginine into L-ornithine and urea. Increased arginase activity resulting in increased synthesis of L-ornithine, which is a precursor of L-proline, is thought to contribute to fibrotic disorders in the lung by promoting collagen production during disease progression [38, 51]. In support of our observation, studies have shown that arginase-1 expression is significantly upregulated in peritoneal macrophages of mice on HFD [52] and in blood mononuclear cells from obese subjects [53]. HIF-1 α is another molecule that not only promotes allergic airway inflammation in mice and is elevated in asthmatics [36] but is also known to induce fibrosis by increasing collagen expression particularly in adipose tissue [37]. While saline-exposed ND fed mice expressed low levels of HIF-1 α in the lungs that were induced only after CRA challenge, baseline HIF-1 α expression in saline-exposed HFD fed mice was inherently high. Further, as in the case of TGF- β 1 and arginase 1, CRA challenge did not significantly alter HIF-1 α expression in HFD fed mice relative to the saline-exposed group.

Interestingly, PGE₂ levels in CRA-challenged HFD fed mice were significantly reduced relative to not only saline-exposed HFD mice but also CRA-challenged ND fed mice. PGE₂, a lipid mediator that is synthesized by virtually all cell types in the lung, is known to induce relaxation of the airways via interaction with the EP2 receptor in mice [54]. It is possible that decreased PGE₂ levels might contribute to the increased AHR associated with asthma in an obese setting. How expression of PGE₂ might be altered after allergen challenge in an obese setting is not entirely clear. One possibility could be PAI-1. There is a marked increase in PAI-1 expression in the lungs of CRA-challenged HFD mice compared to ND fed counterparts. Studies with fibroblasts indicate that activation of plasminogen stimulates PGE₂ release and basal production of PGE₂ in *Plai*^{-/-} fibroblasts was found to be higher than in WT cells [55]. It is conceivable that elevated PAI-1 levels may inhibit plasminogen activation resulting in decreased PGE₂ levels in the lungs of CRA-challenged HFD mice. However, additional studies are needed to confirm this.

Overall, our findings lead us to infer that the underlying mechanisms contributing to pulmonary inflammation after CRA exposure are distinct in ND and HFD fed mice. In ND fed mice, airway inflammation is caused by eosinophilia, elevated inflammatory mediators (cytokines, chemokines, growth factors, etc.), mucus hypersecretion and smooth muscle hypertrophy after allergen challenge that can contribute to the increased AHR. Further, pulmonary edema associated with this inflammatory response can cause loss of surfactant and infiltration of the interstitium by inflammatory cells potentially making the lungs less

elastic. On the other hand, in HFD fed mice with minimal inflammation, factors such as innate (obesity-induced) lung fibrosis due to dysfunction of extracellular matrix metabolism (elevated collagen deposition) that can be attributed to elevated baseline levels of pro-fibrotic factors such as TGF- β , arginase-1 and HIF-1 α along with altered levels of the biological mediators PGE₂ and PAI-1 after allergen exposure are likely to result in impaired lung function. Further, our findings demonstrate that a HFD does not worsen allergic airway inflammation induced by CRA in the present study, although a different allergen (fungal, dust, pollen, etc.) may provoke alternate antigen-specific effects. Overall our studies suggest that HFD can potentiate the development of lung fibrosis that may contribute to poor asthma control in obese individuals.

Acknowledgments

This work was supported by National Institutes of Health grants AI35796 (PS) and DK053189 (DAB), and a University of Minnesota AHC Faculty Research Development Grant (PS and DAB).

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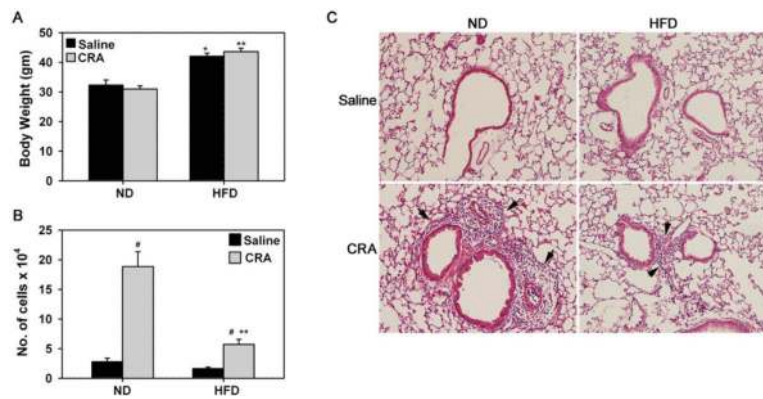
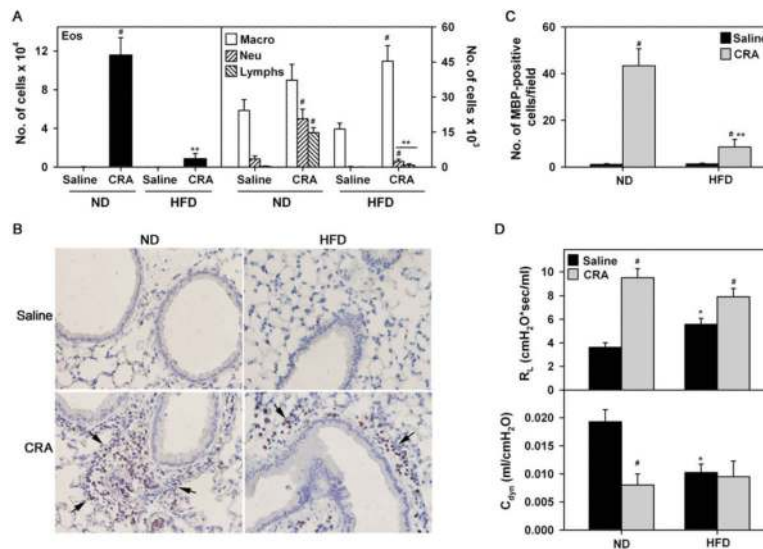
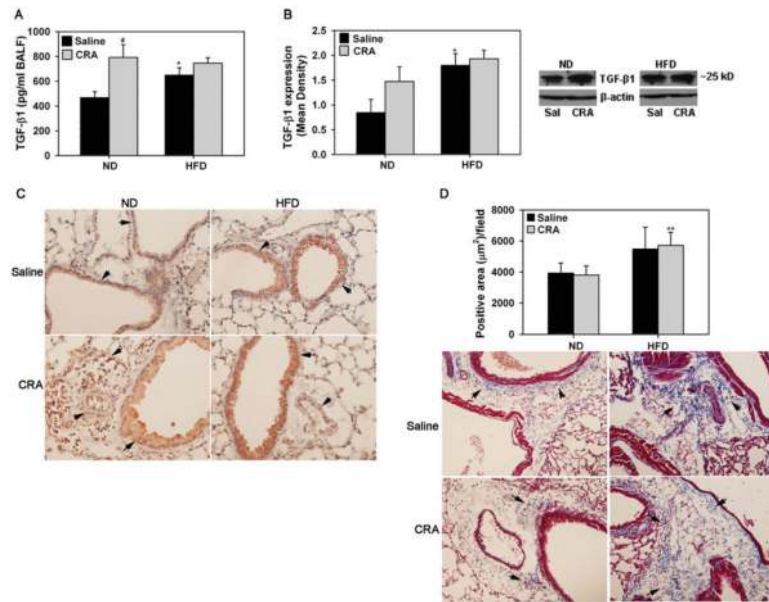


FIGURE 1.

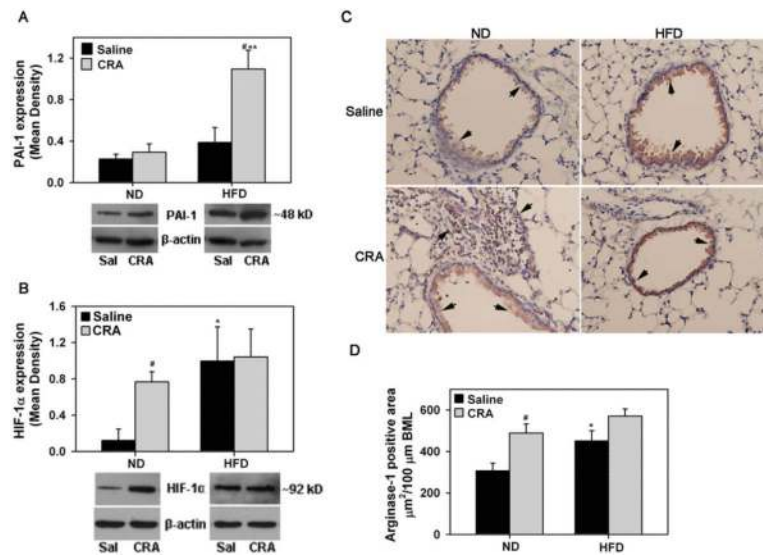
Decreased airway cellular inflammation in HFD fed mice after allergen challenge. Mice weaned at 3 weeks were maintained on a ND or a HFD for 9 weeks and then sensitized and challenged with CRA. Control mice received saline instead of CRA. Mice were maintained on the assigned diets during allergen exposure. (A) Body weight of ND and HFD fed mice measured at the end of allergen challenge. (B) Total cell counts in BALF of CRA-challenged and saline-exposed mice 24 hours after the last challenge. (C) Cellular infiltration of lungs evaluated by H&E staining of paraffin embedded lung sections. Representative images from each group at a magnification of $\times 100$ are shown. Arrows highlight positively stained (purple) inflammatory cells. Data represent mean \pm SEM. Combined data of $n = 6-9$ ND fed and $11-15$ HFD fed mice is shown in A and B. $*P < .01$ in A for comparison of saline-exposed ND and HFD fed mice; $\#P < .001$ in B for comparison of saline versus CRA-challenged mice; $**P < .01$ in A and $P < .001$ in B for comparison of CRA-challenged ND and HFD fed mice.

**FIGURE 2.**

Reduced airway eosinophilia in CRA-challenged HFD fed mice. (A) Differential cell counts in BALF of saline and CRA-challenged ND and HFD fed mice 24 hours after the last challenge. Eosinophil [Eos] counts are shown in the left panel; macrophage [Macros], neutrophil [Neu], and lymphocyte [Lymphs] counts are shown in the right panel. Note difference in scale. (B and C) Lung tissue eosinophils assessed by immunohistochemical staining of sections from saline and CRA-challenged ND and HFD mice with rat mAb against murine MBP. Representative images for each group at a magnification of $\times 200$ are shown. Arrows highlight reddish-brown stained MBP-positive cells. MBP-positive cells in 10–15 randomly selected non-overlapping microscopic fields were counted at a magnification of $\times 400$ and expressed as the average number of cells/field. (D) Measurement of AHR in response to methacholine challenge by invasive plethysmography. R_L and C_{dyn} to inhaled methacholine at 50 mg/mL are shown. Data represent mean \pm SEM. Combined data of $n = 6$ –9 ND fed and 11–16 HFD fed mice in A, 6–7 ND fed mice and 5–10 HFD fed mice in C, and 7–8 ND fed and 6–9 HFD fed mice is shown in D. $^{\#}P < .005$ in ND fed group for Eos, Neu, and Lymphs, $P < .04$ for Neu and $P < .001$ for Macros in HFD fed group in A, $P < .001$ in ND fed group and $P < .03$ in HFD fed group in C, and $P < .01$ in ND and HFD fed group in D, upper panel and $P < .01$ in ND group in D, lower panel for comparison of saline versus CRA-challenged mice; $^*P < .01$ for comparison of saline-exposed ND and HFD fed mice in D; $^{**}P < .002$ in A and C for comparison of CRA-challenged ND and HFD fed mice.

**FIGURE 3.**

HFD fed mice exhibit increased TGF-β1 expression. (A) TGF-β1 levels in BALF from saline and CRA-challenged ND and HFD fed mice by ELISA. (B) TGF-β1 expression in lung tissue of saline and CRA-challenged ND and HFD fed mice by Western blot analysis using polyclonal antibodies against TGF-β1 followed by densitometry. Densitometry data is normalized against expression of β-actin used as an internal control. TGF-β1 expression in lung tissue from a representative mouse for each group is shown adjacent to the bar graph. (C) TGF-β1 expression in lung tissue from saline and CRA-challenged ND and HFD fed mice ($n = 3$ mice for saline group and 5 mice for CRA-challenged group) was detected by immunohistochemistry with polyclonal antibodies against TGF-β1. Representative images from each group at a magnification of $\times 200$ are shown. Arrows highlight structures with positive staining (brown). (D) Total lung collagen was evaluated by Masson's Trichrome staining of lung sections from saline and CRA-challenged ND and HFD fed mice. Trichrome-positive areas (3–8 microscopic fields/slide) were quantitated using Fiji software (an image processing package based on ImageJ). Representative images (magnification $\times 100$) are shown for saline and CRA-challenged groups below bar graph. Arrows highlight areas with positive staining for collagen (blue). Data represent mean SEM. Combined data of $n = 5-7$ mice/group in A and 3–6 mice/group in B is shown. In D, combined data of all fields for each group representing $n = 5-6$ mice for saline group and 7–8 mice for CRA-challenged group is shown. $\#P < .012$ in the ND fed group in A for comparison of saline versus CRA-challenged mice; $*P < .025$ in A and B for comparison of saline-exposed ND and HFD fed mice. $**P < .04$ in D for comparison CRA-challenged ND and HFD fed mice.

**FIGURE 4.**

Expression of PAI-1, HIF-1 α , and arginase-1 in HFD fed mice. PAI-1 (A) and HIF-1 α (B) expression in lung tissue of saline and CRA-challenged ND and HFD fed mice by Western blot analysis using polyclonal antibodies against PAI-1 and HIF-1 α , respectively, followed by densitometry. Densitometry data are normalized against expression of β -actin. PAI-1 and HIF-1 α expression in lung tissue from a representative mouse for each group is shown below bar graph in A and B, respectively. (C) Arginase-1 expression by immunohistochemical staining of lung sections from saline and CRA-challenged ND and HFD fed mice with polyclonal antibodies against arginase-1. Representative images (magnification $\times 200$) are shown for saline and CRA-challenged groups with arrows highlighting arginase-1 positive staining (brown). (D) Quantitation of arginase-1 expression in airway epithelium by ImageJ analysis (5–9 airways/slide). Data represent mean \pm SEM. Combined data of $n = 4$ –6 mice/group is shown in A, B, and D. $\#P < .012$ in the HFD fed group in A and in the ND fed group in B and D for comparison of saline versus CRA-challenged mice; $*P < .05$ in B and $P < .01$ in D for comparison of saline-exposed ND and HFD fed mice; $**P < .012$ in A for comparison of CRA-challenged ND and HFD fed mice.

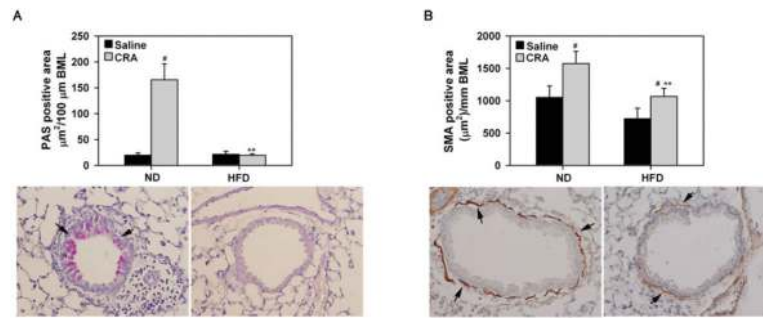


FIGURE 5.

Decreased mucus secretion and smooth muscle mass in lungs of HFD fed mice after CRA challenge. (A) Airway epithelial mucus secretion was examined after PAS staining of lung tissue sections from saline and CRA-challenged ND and HFD fed mice. PAS-positive area in airways (3–13 airways/slide with perimeter $700 \pm 21.3 \mu\text{m}$) was quantitated by ImageJ analysis of captured images. Representative images (magnification $\times 200$) with arrows highlighting PAS-positive staining (pink) in airways are shown for CRA-challenged groups below bar graph. (B) Area of the smooth muscle layer was quantitated based on immunohistochemical staining for α -SMA in lung sections from the above groups of mice followed by ImageJ analysis of captured images (4–17 airways/slide with perimeter $676.6 \pm 16.3 \mu\text{m}$). Representative images (magnification $\times 200$) are shown for CRA-challenged groups below bar graph with arrows highlighting airway α -SMA-positive cells (brown). Data represent mean \times SEM. In A and B, combined data of all airways for each group representing $n = 3\text{--}4$ mice for saline group and $5\text{--}6$ mice for CRA-challenged \pm group is shown. [#] $P < .01$ in the ND fed group in A, $P < .03$ in the ND fed group and $P < .05$ in the HFD fed group in B for comparison of saline versus CRA-challenged mice; ^{*} $P < .01$ in A, and $P < .02$ in B for comparison of CRA-challenged ND and HFD fed mice.

TABLE 1

Lung Cytokine, Chemokine, and Eicosanoid levels in CRA-challenged HFD and ND fed mice

Mediator	ND		HFD	
	Saline	CRA	Saline	CRA
IFN γ^a	0.13±0.15	1.36±0.63	0.32±0.58	0.0±0.0
IL-2 ^a	3.88±0.6	16.76±11.58	5.81±0.32	4.66±0.46
IL-4 ^a	3.35±0.53	3.66±0.7	5.04±0.28	4.0±0.38
IL-5 ^a	1.79±0.27	62.8±22.49 [#]	2.36±0.16	4.62±1.53 ^{**}
IL-13 ^a	2.0±0.68	208.33±63.47 [#]	2.0±0.53	2.0±0.34 ^{**}
TNF- α^a	2.04±2.48	23.22±7.48 [#]	0.5±0.5	0.5±0.5 ^{**}
CCL11 ^b	17.3±7.45	72.29±7.04 [#]	11.08±3.3	8.61±5.2 ^{**}
CCL2 ^b	48.27±2.64	77.12±7.89 [#]	25.0±5.71	23.25±2.7 ^{**}
LTC ₄ ^c	3.24±1.05	40.69±18.72 [#]	2.42±0.72	1.78±0.86 ^{**}
PGE ₂ ^c	53.12±29.17	59.51±17.5	57.91±18.29	14.87±4.79 ^{#**}

^a pg/mg protein in lung tissue,^b pg/ml BALF,^c ng/g lung tissue.[#] $P < .03$ for IL-5 and IL-13, $P < .02$ for TNF- α , $P < .01$ for CCL11 and CCL2 and $P < .04$ for LTC₄ in ND fed mice and $P < .03$ for PGE₂ in HFD fed mice for comparison of saline versus CRA-challenged groups;^{**} $P < .03$ for IL-5, $P < .02$ for IL-13 and PGE₂, $P < .01$ for TNF- α , $P < .001$ for CCL11 and CCL2, and $P < .04$ for LTC₄ for comparison of CRA-challenged HFD versus ND mice.