

RESEARCH ARTICLE

Therapeutic ketosis decreases methacholine hyperresponsiveness in mouse models of inherent obese asthma

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Abstract

Obese asthmatics tend to have severe, poorly controlled disease and exhibit methacholine hyperresponsiveness manifesting in proximal airway narrowing and distal lung tissue collapsibility. Substantial weight loss in obese asthmatics or in mouse models of the condition decreases methacholine hyperresponsiveness. Ketone bodies are rapidly elevated during weight loss, coinciding with or preceding relief from asthma-related comorbidities. As ketone bodies may exert numerous potentially therapeutic effects, augmenting their systemic concentrations is being targeted for the treatment of several conditions. Circulating ketone body levels can be increased by feeding a ketogenic diet or by providing a ketone ester dietary supplement, which we hypothesized would exert protective effects in mouse models of inherent obese asthma. Weight loss induced by feeding a low-fat diet to mice previously fed a high-fat diet was preceded by increased urine and blood levels of the ketone body β -hydroxybutyrate (BHB). Feeding a ketogenic diet for 3 wk to high-fat diet-fed obese mice or genetically obese *db/db* mice increased BHB concentrations and decreased methacholine hyperresponsiveness without substantially decreasing body weight. Acute ketone ester administration decreased methacholine responsiveness of normal mice, and dietary ketone ester supplementation of high-fat diet-fed mice decreased methacholine hyperresponsiveness. Ketone ester supplementation also transiently induced an “antiobesogenic” gut microbiome with a decreased *Firmicutes/Bacteroidetes* ratio. Dietary interventions to increase systemic BHB concentrations could provide symptom relief for obese asthmatics without the need for the substantial weight loss required of patients to elicit benefits to their asthma through bariatric surgery or other diet or lifestyle alterations.

asthma; β -hydroxybutyrate; ketogenic diet; mice; obesity

INTRODUCTION

Asthma is a common, chronic pulmonary disorder that while diverse in presentation typically involves a complex interaction of airway reactivity and inflammation (1). Obesity is highly prevalent among asthmatics (2). Whereas about one-third of the U.S population is obese, the prevalence of obesity among asthmatics is $\geq 50\%$ (3, 4), and asthma is among the most important comorbidities of obesity (5). Obese asthmatics respond poorly to standard therapy (6) and are hospitalized for their asthma at an increased rate relative to normal weight asthmatics (7). Obesity alters responsiveness to bronchoconstricting agents, making both humans and mice hyperresponsive to the most common clinically used asthma diagnostic, methacholine (8–11). Metabolic reprogramming, oxidative stress, and altered immune status all contribute to obese asthma and are considered mediators of lung dysfunction (2, 12–14). Obese asthma exists as phenotypes termed “inherent” and “allergic,” both of which can be effectively modeled in mice. Whereas allergic asthma typically manifests in large

airway hyperresponsiveness reactivity (AHR), obese asthma involves both proximal and distal lung compartments (15–17). Animal models suggest that obesity causes an inherent asthma-like phenotype involving proximal and distal lung compartments (18) through innate immune pathways, with the same mediators (e.g., TNF, IL-1 β , and IL-17A) augmenting pathologic outcomes in obese allergic asthma (19–23). Altered immune responses and changes in pulmonary structure and function are evident in obese asthma patients, with TNF, macrophages (21), adipokines (24–26), and altered cellular metabolism (especially mitochondrial metabolism) (27–34) emerging as important modulators. Elevated markers of oxidative stress are found in obese asthmatics and are believed to contribute to the pathogenesis of obese asthma (35, 36).

Obesity is fundamentally a state of altered metabolism (37). In obese asthmatics, adipose tissue preferentially stores fatty acids instead of liberating them (38) and is more proinflammatory than adipose tissue from obese nonasthmatics (39). Weight loss reduces several comorbidities associated with obesity (40–42), including the hyperresponsiveness to

inhaled methacholine manifest in inherent and allergic asthma (8, 11, 12, 18, 43–45). In humans, 5–10% body weight loss is typically required to promote benefit (defined as a minimal clinically important difference) in obese asthmatics (46), whereas body weight loss in mouse models of obese asthma is typically much more substantial, approaching the weights of normal-weight mice (18). However, reducing caloric intake, increasing energy expenditure, and pharmacological interventions as means of weight loss in human populations have limited long-term success due to poor long-term compliance, a lack of desirability, and high cost that limit their success as treatments for obese asthma. Bariatric surgery is the most effective weight loss intervention in the morbidly obese and is also an effective therapy for the comorbidities of obesity (11, 40–43, 47–49). Ketone bodies are elevated during weight loss (50–52), including for a brief period following bariatric surgery (53), and can modulate several of the key pathological processes involved in obese asthma (54–56). Despite our reports on the benefits of bariatric surgery, especially for nonallergic obese asthmatics (6, 8, 11, 39, 43, 47, 57–60), the limited success of this intervention on lung function in obese allergic asthmatics, and the poor long-term weight loss achieved by lifestyle alterations, highlight the continued need for specific treatments for obese allergic asthma.

During weight loss, fatty acids mobilized from adipose tissue are catabolized in the liver to the ketone bodies acetoacetate (AcAc) and β -hydroxybutyrate (BHB). Whereas circulating ketone body concentrations are normally repressed after eating, they become elevated during events that promote weight loss, including calorie restriction and exercise (51). Ketone bodies provide an energy source that makes cells less reliant on glycolysis (61). Ketone bodies also function as antioxidants (54, 55) and exert anti-inflammatory effects, including inhibition of the NLRP3 inflammasome and subsequent IL-1 β production (56, 62), which are implicated in the pathogenesis of allergic asthma and obese asthma in mice fed a high-fat diet (20, 56, 62, 63). In addition, elevated BHB levels induced through alternate day caloric restriction are correlated with reductions in oxidative stress and inflammation, along with improved clinical findings, in overweight asthmatic subjects (64). Importantly, augmenting ketone bodies in humans is well tolerated (65).

Despite the strong connections between the mechanisms underlying obese asthma and the beneficial effects of ketone bodies, their potential to be used therapeutically in obese asthma has not been evaluated. We hypothesized that since weight loss elicits increases in ketone bodies that can exert significant anti-inflammatory, redox-regulating, and metabolic effects, ketone bodies could be relevant tools for treating obese inherent asthma. Our objective was to evaluate the effectiveness of augmenting ketone body concentrations on diminishing pathological features of obese asthma in mouse models and to provide mechanistic insight into the means by which benefit may be induced. Further understanding the efficacy and mechanisms of ketone bodies could provide new pharmacological targets for treatment or prevention of obese asthma, as could be addressed in subsequent clinical trials.

MATERIALS AND METHODS

Study Approval

Animal experiments were reviewed and approved by the University of Vermont's Institutional Animal Care and Use Committee (protocol nos. 16-041, 18-023, and PROTO202000195), in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (revised 2011). Studies involving potentially hazardous materials were reviewed and approved by the University of Vermont's Institutional Biosafety Committee (protocol nos. 09-018 and REG-201900052).

Mice

Wild-type C57BL/6J mice (stock no. 000664) were purchased from The Jackson Laboratory (Bar Harbor, ME). *Lepr^{db/+}* mice on the C57BL/6J background purchased from The Jackson Laboratory (stock number 000697) were bred at the University of Vermont. Age-matched, female, normal-weight *Lepr^{+/+}* and obese *Lepr^{db/db}* offspring were used for studies. All animals were maintained on 12-h light-dark cycle and were provided chow (~15% kcal from fat; TestDiet, St. Louis, MO) and water ad libitum in an American Association for the Accreditation of Laboratory Animal Care-accredited facility. Low-fat diet (D12450B), high-fat diet (D12492), and ketogenic diet (D03022101) were purchased from Research Diets (New Brunswick, NJ) and stored frozen. Food in cages was replaced twice each week. Mice were euthanized with sodium pentobarbital (150 mg/kg by intraperitoneal injection; Wilcox Pharmacy, Rutland, VT).

High-Fat Diet Model of Obese Inherent Asthma and Diet-Induced Weight Loss

Twenty-one-week-old male C57BL/6J mice were purchased from The Jackson Laboratory having been maintained on food containing 10% fat (low-fat diet; LFD) or 60% fat (high-fat diet; HFD) from 6 wk of age. Mice were acclimated at the University of Vermont for a week following shipping and were maintained on their same food or subjected to dietary weight loss regimens. Obese mice were switched from the HFD to the LFD (HFDtoLFD), periodically assessed for body weight and ketone concentrations in urine or blood, and maintained on the LFD for a time sufficient to induce significant and substantial weight loss compared with the matched HFD mice that continued to gain weight, which was typically 2–3 wk and making the weight of the HFDtoLFD mice indistinguishable from the LFD-fed mice.

Genetic Model of Obese Inherent Asthma

Twenty-week-old male and female *Lepr^{db/db}* mice and their lean control littermates were maintained on normal chow (NC) and then randomly divided into groups to be switched to the ketogenic diet (KD) or kept on NC. The mice were maintained on these diets for 3 wk until they were assessed for pulmonary responsiveness to inhaled methacholine.

Ketone Ester Supplementation of Mouse Diets

Twenty-week-old male C57BL/6J mice were purchased from The Jackson Laboratory having been maintained on LFD or HFD from 6 wk of age. After 1 wk of acclimation at the University of Vermont, the mice were switched to HFD or LFD containing 20% wt/vol water for 1 wk. Some of the obese mice were then switched to HFD containing 20% w/v ketone ester (KE; HVMN Inc., San Francisco, CA) and maintained on these diets for 3 wk until they were assessed for pulmonary responsiveness to inhaled methacholine. The ketone ester contains 25 g of pure ketones per 65 mL and less than 2% stevia leaf extract, flavorings, and preservatives.

Ketone Ester Gavage

Twenty-week-old C57BL/6J mice were gavaged with 200 μ L of KE, and BHB levels were measured in blood collected from the submandibular vein using a ketone meter (Keto-Mojo, Napa, CA). Mice gavaged with 200 μ L of KE or water were rested for a half hour then were assessed for acute effects of KE on pulmonary responsiveness to inhaled methacholine ($\sim$ 1 h following water or KE gavage, accounting for time needed for mice to reach a steady anesthetic plane and be baselined on the flexiVent).

Assessment of Pulmonary Responsiveness to Methacholine

Responsiveness to inhaled methacholine was assessed in closed-chested mice. The mice were anesthetized with intraperitoneal sodium pentobarbital (90 mg/kg), the trachea was cannulated with a blunted 18 g needle, and the mice were connected to a flexiVent computer controlled small animal ventilator (SCIREQ, Inc., Montreal, Canada). The mice were ventilated at 200 breaths/min with a 0.25-mL tidal volume and 3-cmH₂O positive end-expiratory pressure (PEEP). Next, the mice were paralyzed with an intraperitoneal injection of pancuronium bromide (0.8 μ g/kg). The mice were stabilized over $\sim$ 10 min of regular ventilation at a PEEP of 3 cmH₂O. A standard lung volume history was then established by delivering two total lung capacity maneuvers to a pressure limit of 25 cmH₂O and holding for 3 s. Next, two baseline measurements of respiratory input impedance (Z_{rs}) were obtained from 2-s multifrequency oscillations at PEEP = 3 cmH₂O. This was followed by an inhalation of aerosolized PBS (control) for 10 s, achieved by an in-line piezo electric nebulizer (Aeroneb, Aerogen, Galway, Ireland). Z_{rs} was then measured every 10 s for 3 min (18 measurements of Z_{rs} in total). This complete sequence of maneuvers and measurements was then repeated for aerosol exposures to saline and three ascending doses of aerosolized methacholine (12.5, 25, and 50 mg/mL). Data were fit to the constant phase model of the lung (66) to provide values reflecting airway resistance (R_N), tissue damping (G), and tissue elastance (H). Individual data points were excluded when the coefficient of determination to the constant phase model was below 0.85 or when values were below baseline levels. All data points for a particular dose of methacholine in an individual mouse were excluded when $>50\%$ or more of individual data points (>9 of 18) were excluded. The mean values $\pm$ SE of R_N , G ,

and H in each of the mouse groups, at each incremental methacholine dose, are reported.

Serum Collection and Analysis

Following euthanasia at the completion of flexiVent analysis, $\sim$ 300 μ L of blood were collected via cardiac puncture from the right ventricle using a 25-g needle attached to a 1-mL syringe, transferred into serum separator tubes (Becton Dickinson, Franklin Lanes, NJ) and centrifuged, and serum was kept frozen at -80°C . Serum was used to determine the concentration of beta-hydroxybutyrate (BHB) (BHB colorimetric assay kit no. 700190; Cayman Chemical), 8-isoprostane (8-isoprostane ELISA kit; Cayman Chemical), and L-lactate [L-lactate assay kit II (Eton Bioscience) following deproteinization using Strata-Clean resin (Agilent, Santa Clara, CA)].

Bronchoalveolar Lavage Collection and Processing

Anesthetized mice, following pulmonary function assessment in some studies, were lavaged through an 18-gauge tracheal cannula with 1 mL of room temperature DPBS (Sigma-Aldrich, St. Louis, MO). Cells were manually counted immediately in white blood cell stain (0.2 mg/mL crystal violet in 2% acetic acid) using a hemocytometer, the lavage fluid was centrifuged at 400 g for 10 min at room temperature, and cell-free supernatants were frozen at -80°C for analysis. Cell pellets were resuspended in saline and mounted on slides by cytospin (100,000 cells per slide) for hematoxylin and eosin (H&E) staining and differential analysis.

Urine Collection and Analysis

Urine was collected from mice by scruffing them over parafilm and waiting for voluntary urination. $\sim$ 50 to 100 μ L of urine was collected from each mouse and transferred to a tube with a pipette and frozen at -80°C until analysis. Urine was later used to determine the concentration of BHB using a commercial colorimetric assay (BHB colorimetric assay kit no. 700190, Cayman Chemical).

Quantitative RT-PCR

Following the collection of BAL fluid, lungs were dissected, ground to a fine powder using a liquid nitrogen-chilled mortar and pestle, and stored at -80°C until analysis. Total RNA was extracted from an aliquot of the snap-frozen and pulverized lung lobes using the PrepEase RNA Isolation kit (USB Corp., Cleveland, OH) and reverse transcribed to cDNA using the iScript kit (Bio-Rad, Hercules, CA). Primers were designed for mouse genes using NCBI Primer-BLAST (RRID:SCR_003095) and synthesized by Integrated DNA Technologies (Coralville, IA). Quantitative RT-PCR was performed using SYBR Green Supermix on a CFX96 or Chromo4 thermocycler using CFX Manager or Opticon Monitor software (Bio-Rad) and normalized to *Rpl13a* using the $\Delta\Delta C_T$ method (Table 1).

Measurement of Fecal Bacterial Diversity

From fecal pellets collected at the time of euthanasia, bacterial DNA was extracted (Qiagen) and subjected to quantitative PCR analysis of the 16S ribosomal RNA (rRNA) present in all eubacteria as well as the 16S rRNA

Table 1. Primers used for RT-qPCR analysis of lung gene expression

Gene	Name	Forward Primer	Reverse Primer
<i>Acta2</i>	Actin alpha 2	TGTGCTGGACTCTGGAGATG	GAAGGAATAGCCACGCTCAG
<i>Actg</i>	Actin gamma	GGATCGGTGGCTCCATTCTG	TGAGGTGTGTACATTTGCCAG
<i>Adipoq</i>	Adiponectin	TGTTCTCTTAATCCTGCCCA	CCAACCTGCACAAGTTCCTT
<i>Ccl2</i>	Chemokine (C-C motif) ligand 2	TCCCTGTCATGCTTCTGGGC	GTTCTGATCTCATTTGGTTCCGATCC
<i>Clca1</i>	Chloride channel accessory 1	AAGCAAACCACTCCCATGAC	TGCGAAAGCATCAACAAGAC
<i>Col1a1</i>	Collagen type I alpha 1	GAGCGAGAGTACTGGATCG	GTTCTGGGCTGATGTACCAGT
<i>Il1b</i>	Interleukin 1 beta	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCTG
<i>Il6</i>	Interleukin 6	CCGGAGAGGAGACTTACAG	GAGCATTGGAAATGGGGTA
<i>Il17a</i>	Interleukin 17 A	CTGCTGAGCCTGGCGGCTAC	GGCGGCACTGAGCTTCCCAG
<i>Muc5ac</i>	Mucin 5 subtype AC	CCATGCAGAGTCTCAGAACAA	TTACTGGAAAGGCCCAAGCA
<i>Muc5b</i>	Mucin 5 subtype B	ACACATGCACCTGCCTCTCTGA	TCCATGGAGTACTTGGATATTC
<i>Myh11</i>	Myosin heavy polypeptide 11	CTCTGGCCTCTCTGTGTGG	TCTTCTTGCCCTTGTGGGA
<i>Myod</i>	Myogenic differentiation 1	GCCGCTGAGCAAAGTGAATG	CAGCGGTCCAGGTGCGTAGAAG
<i>Myog</i>	Myogenin	CTACAGGCCTTGCTCAGCTC	ACGATGGACGTAAGGGAGTG
<i>Nlrp3</i>	NRL family pyrin domain containing 3	ATGCTGCTTCGACATCTCCT	AACCAATGCGAGATCCTGAC
<i>Rpl13a</i>	Ribosomal protein L13a	CCCTCCACCCTATGACAAGA	CTGCCTGTTCCGTAACCTC
<i>Saa3</i>	Serum amyloid A3	CAGGATGAAGCCTTCCATTG	CATGACTGGGAACAACAGGA
<i>Tagln</i>	Transgelin	GCGGCCTTTAAACCCCTCA	CTCCACTAGTCGCTCCCA
<i>Tjp1</i>	Tight junction protein 1	CCACCTCTGTCCAGCTCTTC	CACCGGAGTGATGGTTTCT
<i>Tnfa</i>	Tumor necrosis factor	TCCAGGTTCTCTTCAAGGGA	GGTGAGGAGCACGTAGTCGC
<i>Vim</i>	Vimentin	TGCTTCAAGACTCGGTGGAC	AAGCGCACCTTGTCGATGTA

present specifically in the flora of the mouse gastrointestinal tract, including the phyla *Bacteroidetes* (*Bacteroides*, mouse intestinal *Bacteroides*), *Firmicutes* (*Clostridium perfringens*, *Eubacterium rectale*/*Clostridium coccoides*, and *Lactobacillus/Lactococcus*), and *Proteobacteria* (*Enterobacteriaceae*, *Salmonella*, and *Helicobacter pylori*) using primers described previously (67). Standards were generated from the pooled products of mixed mouse fecal samples following 20 rounds of PCR, which were subsequently used to establish standard curves for each of the primer reactions over a range of 4-fold dilutions between 1:100 and 1:400,000. Cycle threshold values for each PCR reaction were applied to the respective standard curve, run on each plate on a Bio-Rad CFX96 or Chromo4 96-well quantitative real-time PCR detection system. PCR product abundance was log transformed and the average abundance in the low-fat diet group was used to normalize relative abundance from each animal, values that were then used to calculate the means $\pm$ SE for each bacterial 16S rRNA in each mouse group. The average relative abundance of specific 16S rRNAs of each phyla in each mouse were used to calculate the overall *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* abundance, from which the *Firmicutes*:*Bacteroidetes* ratios were calculated.

Data Acquisition, Data Availability, and Statistical Analysis

All experiments involved multiple mice per group and were replicated. The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. Data were analyzed by two-tailed unpaired *t* test or one-way or two-way ANOVA and Bonferroni, Tukey, or Dunnett's multiple comparisons post hoc test using GraphPad Prism 9.1.2 for Windows (GraphPad Software, Inc., La Jolla, CA; RRID: SCR_002798). Data are presented as means $\pm$ SE. *P* < 0.05 in the *t* test or multiple comparisons post hoc test was

considered statistically significant. Significance levels of the tested comparisons are indicated in the figure legends.

RESULTS

Elevated Ketone Body Concentration Precedes Diet-Induced Weight Loss in Obese Mice Previously Fed a High-Fat Diet

The high-fat diet-induced obesity model most closely replicates the findings of weight gain and the development of metabolic syndrome common in obese humans (68, 69). We have previously reported that diet-induced weight loss in obese mice previously fed a high-fat diet decreases methacholine hyperresponsiveness (18) and promotes visceral adipose tissue homeostasis (70). To examine the temporal relationships between body weight and BHB levels, C57BL/6J mice were maintained on low-fat diet (LFD) or high-fat diet (HFD) until the latter were markedly obese. Then, a cohort of the high-fat diet-fed mice were provided the low-fat diet for 3 wk (HFDtoLFD) while other mice were maintained on their original diets. Whereas body weights of the HFDtoLFD mice did not differ significantly from the HFD group until day 9 (Fig. 1A), urinary BHB levels were significantly elevated on days 2 and 7 in the HFDtoLFD mice (Fig. 1B), with levels declining to near baseline at day 9 and beyond. At the end of the study, visceral adipose tissue (VAT) weight was significantly decreased in the HFDtoLFD mice to levels equivalent to those of the LFD mice (Fig. 1C), whereas serum BHB concentrations were the same across the groups at the end of the study (means $\pm$ SD for LFD = 0.25 ± 0.01 mM, HFD = 0.27 ± 0.02 mM, and HFDtoLFD = 0.27 ± 0.02 mM; *P* = 0.29). In a separate cohort of HFDtoLFD mice, blood BHB levels were significantly elevated over baseline on days 1, 2, and 3 after switching from the high-fat to the low-fat diet and, although still significantly elevated on day 6, were returning to baseline levels, which were low again on day 9 (Fig. 1D).

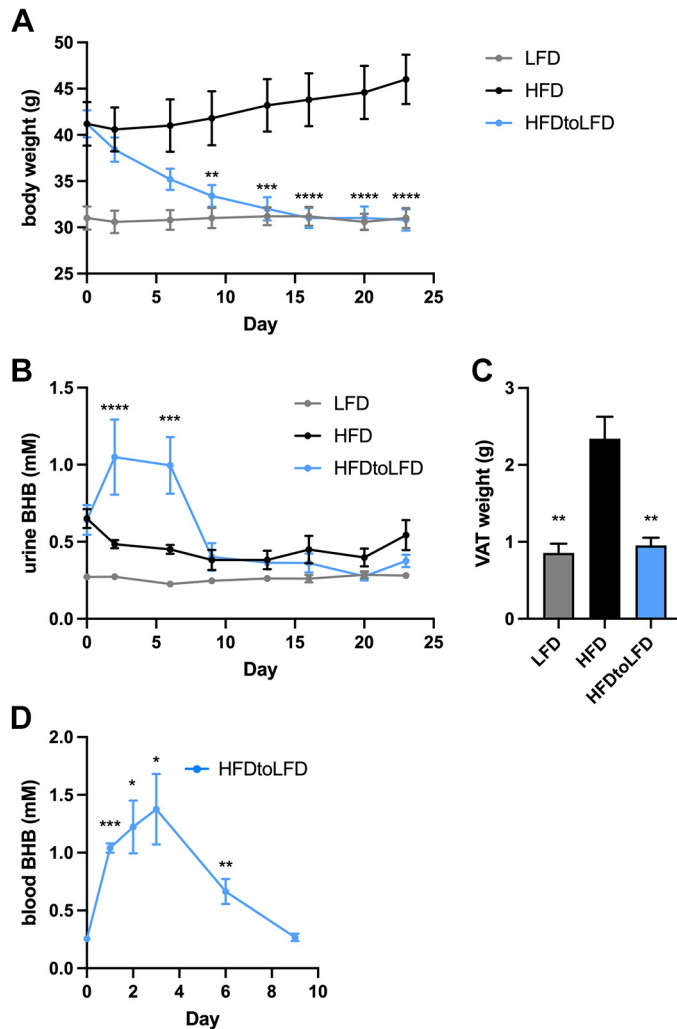


Figure 1. High-fat diet-induced obese mice switched to a low-fat diet produce β -hydroxybutyrate preceding weight loss. Mice were fed a low-fat diet (LFD) or a high-fat diet (HFD) for 16 wk and then either maintained on the same diets or switched (day 0) from the HFD to a LFD (HFDtoLFD) for 3 additional weeks. Body weight (A), urine β -hydroxybutyrate (BHB) (B), and visceral adipose tissue (VAT) weight at day 23 (C) were measured. In a separate cohort of HFDtoLFD mice, blood β -hydroxybutyrate (BHB) was measured daily (D); $n = 5$ mice/group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, comparing HFDtoLFD and HFD (A and B), compared with HFD group (C), or compared with day 0 (D).

Feeding a Ketogenic Diet to Diet-Induced Obese Mice Modestly Decreases Body Weight but Substantially Increases Serum BHB Concentrations and Decreases Methacholine Hyperresponsiveness

A ketogenic diet is one in which the fat content is elevated beyond that in the HFD at the expense of reducing carbohydrates and in which circulating ketone body levels are elevated. Increasing dietary fat content causes “therapeutic ketosis” in the absence of fasting and induces a unique metabolic state in mice that results in weight loss of similar magnitude to that of caloric restriction (71). As elevations in ketone body concentrations precede diet-induced weight loss, we examined whether providing a high-fat (80% of calories), low-carbohydrate (<0.1% of calories) ketogenic diet

(KD) to obese mice previously fed a high-fat diet would attenuate methacholine hyperresponsiveness to a magnitude similar to that elicited by feeding a low-fat diet (18). Mice were maintained on LFD or HFD until the HFD-fed mice were significantly and substantially obese. Mice were then maintained on these diets, or a cohort of the HFD-fed mice were either provided the LFD (HFDtoLFD) or the KD (HFDtoKD) for 3 wk. Whereas feeding the LFD to HFD-induced obese mice substantially decreased body weight to levels similar to the LFD mice, obese mice fed the KD only modestly decreased body weight (Fig. 2A) and had significantly elevated levels of serum BHB (Fig. 2B) compared with the HFD-fed mice. Bronchoalveolar lavage cellularity was not significantly different between the groups (Fig. 2C), and all cells were identified as alveolar macrophages by H&E staining. Assessment of inhaled methacholine responsiveness, a defining criterion of obesity-associated asthma (12, 18, 47), showed significantly decreased central airway (Newtonian) resistance (R_N), tissue damping (G), and tissue elastance (H) in mice switched from the HFD to either LFD or KD (Fig. 2, D and E).

Feeding a Ketogenic Diet to Genetically Obese Mice Substantially Increases Serum BHB Concentrations and Decreases Methacholine Hyperresponsiveness of the Central Airways

The leptin “resistant” *db/db* mice on a C57BL/6J background are a genetic model of hyperphagic obesity in which mice are deficient in the long form of the leptin receptor (ObRb), conferring impaired signaling along the canonical pathway in response to leptin (72), much like what is observed functionally in obese humans and accompanied by other manifestations of metabolic syndrome. Studies in *db/db* mice demonstrate obesity-associated defects in both the innate and adaptive immune responses (73) and inherent methacholine hyperresponsiveness when obese (74). Wild-type (WT) and homozygous *Lepr^{db/db}* (DbDb) mice were maintained on normal chow, and then cohorts from each strain were switched to KD or maintained on normal chow for 3 wk. WT mice remained normal weight on either food, whereas the DbDb mice were markedly obese and slightly increased their weight on the KD (Fig. 3A). In both WT and DbDb mice, feeding the KD significantly and substantially increased serum BHB levels at the end of the study (Fig. 3B), whereas there was no effect of the type of food consumed on BAL cells (Fig. 3C), all of which were alveolar macrophages. DbDb mice exhibited profound methacholine hyperresponsiveness, with significant increases in central airway resistance and tissue elastance (Fig. 3, D–F). DbDb mice fed the KD showed significantly decreased central airway resistance in response to the highest dose of inhaled methacholine compared with DbDb mice fed normal chow.

Substantially Elevating Serum BHB Levels Decreases Methacholine Responsiveness

Since ketone ester supplementation in the food decreased methacholine hyperresponsiveness in diet-induced and genetic models of obesity while slightly, albeit significantly,

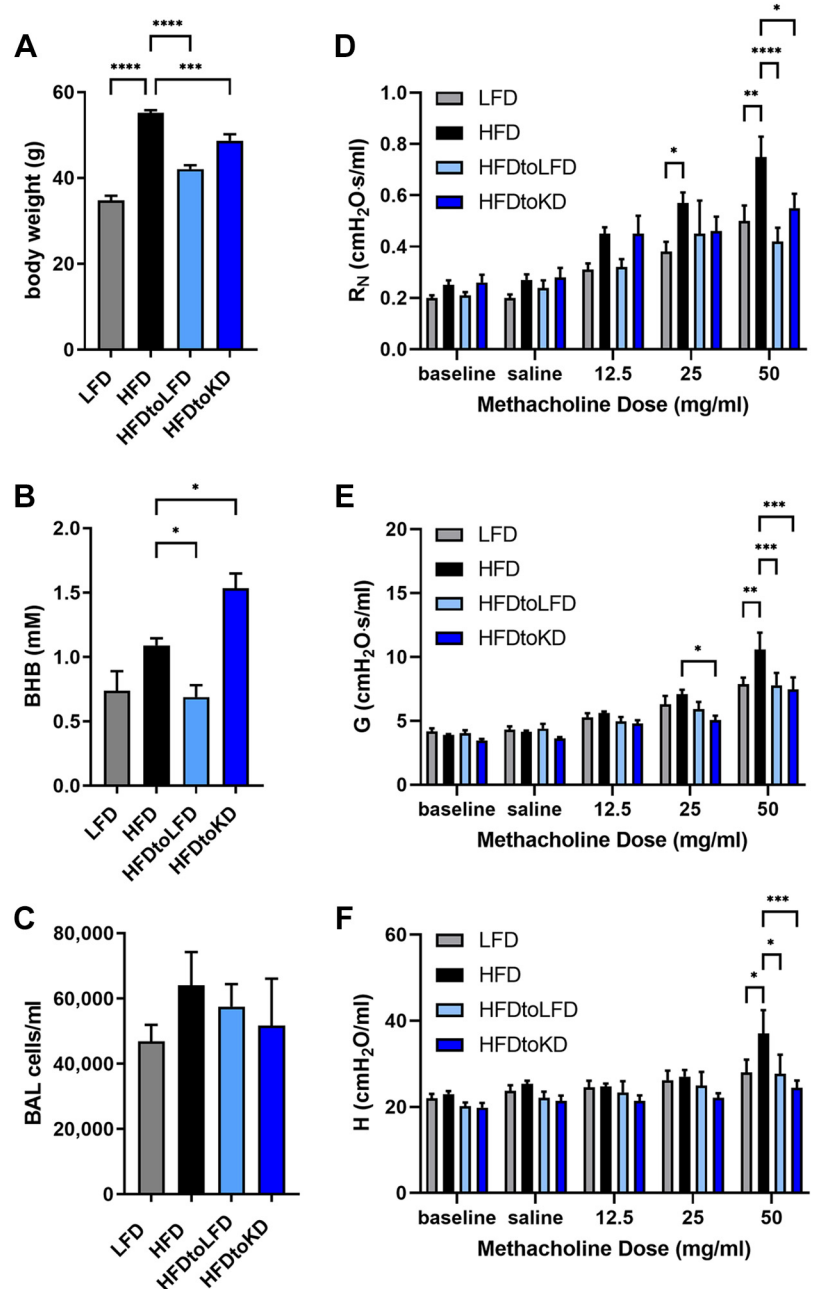


Figure 2. High-fat diet-induced obese mice switched to low-fat or ketogenic diets exhibit decreased methacholine hyperresponsiveness. Mice were fed a low-fat diet (LFD) or a high-fat diet (HFD) for 16 wk and then either maintained on the same diets or switched from the HFD to a LFD (HFDtoLFD) or ketogenic diet (HFDtoKD) for 3 additional weeks. Body weight (A), serum β -hydroxybutyrate (BHB) (B), and total bronchoalveolar lavage (BAL) cells (C) were measured. Airway resistance (R_N) (D), tissue damping (G) (E), and tissue elastance (H) (F) were measured; $n = 8$ –10 mice/group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, compared with indicated group.

increasing serum BHB levels at the end of the studies, we sought to determine the impact of substantially elevated circulating BHB levels during methacholine challenge. Mice fed normal chow were administered by oral gavage 200 μ L of water or an equal volume containing 80 mg ketone ester. Blood BHB levels were substantially elevated above 7 mM at 30 min and 1 h and then began to drop by 2 h, with levels falling to baseline by 6 h (Fig. 4A). A separate cohort of mice were administered water or KE by gavage then assessed for methacholine responsiveness 1 h later, at the time of peak circulating BHB levels. Compared with the water control mice, mice acutely administered KE displayed significantly decreased methacholine responsiveness, especially in tissue damping and tissue elastance (Fig. 4, B–D). As BHB has been

reported to exert antioxidant properties (54, 55, 75, 76), serum levels of 8-isoprostanes, a stable product of lipid peroxidation elevated in exhaled breath of obese asthmatics (14), were measured. The levels of serum 8-isoprostanes present in the mice administered ketone ester by gavage approximately 1 h earlier were significantly less than those in mice administered water (Fig. 4E). Since BHB can be used as an energetic substrate to provide ATP as an alternative to utilizing dietary carbohydrates (e.g., glucose) (77, 78), and since glucose metabolism generates lactate that has been implicated as a driver of asthma pathogenesis (79–81), serum lactate was also measured. In mice administered ketone ester, the serum lactate levels were significantly and substantially decreased (Fig. 4F).

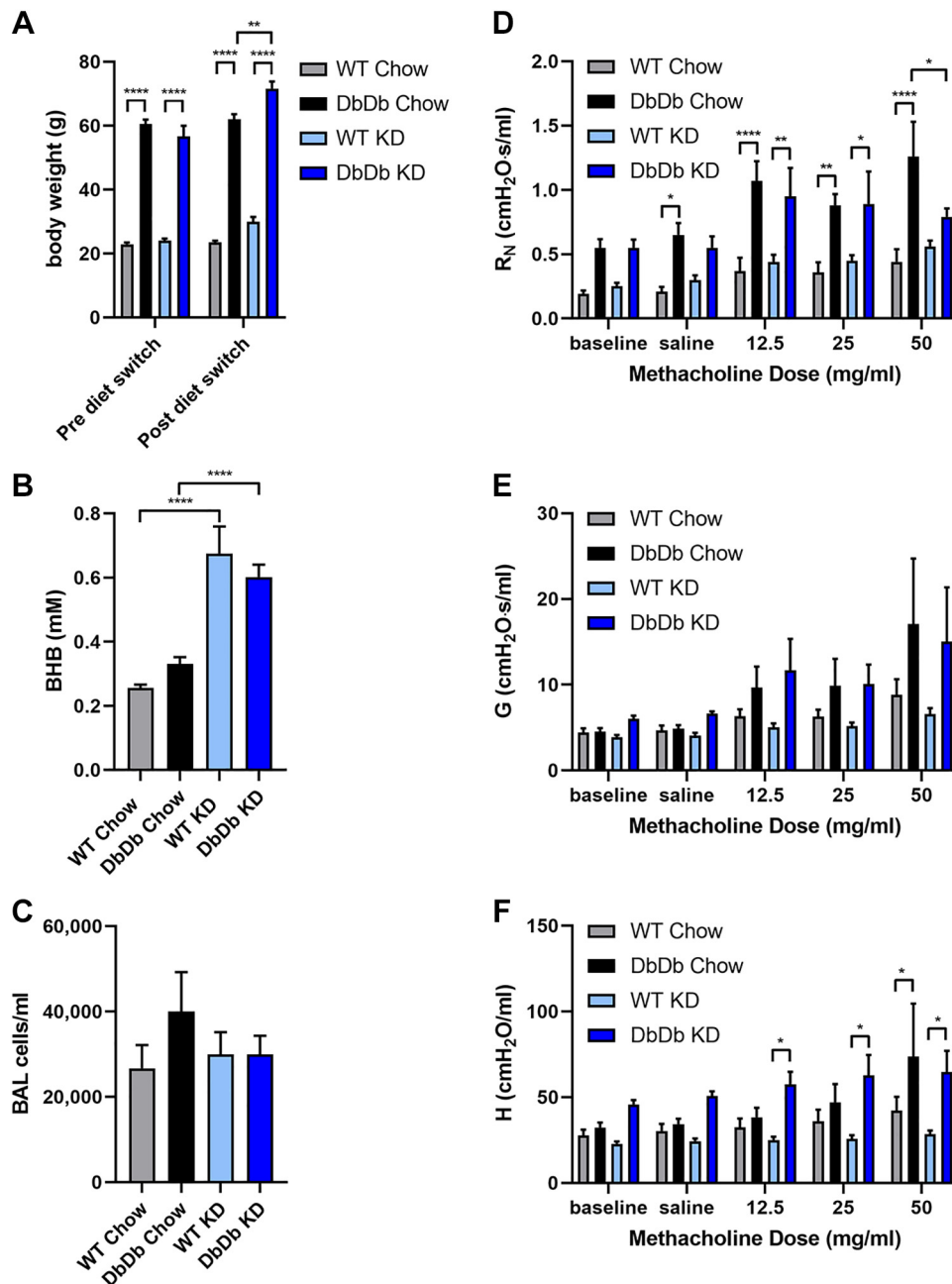


Figure 3. Genetically obese mice fed a ketogenic exhibit decreased methacholine hyperresponsiveness. Wild-type (WT) or *Lep^{db/db}* (DbDb) mice were maintained on a normal diet (Chow) or switched to a ketogenic diet (KD) for 3 wk. Body weight was measured pre- and postswitching diets (A). Serum β -hydroxybutyrate (BHB) (B), total bronchoalveolar lavage (BAL) cells (C), airway resistance (R_N) (D), tissue damping (G) (E), and tissue elastance (H) (F) were measured at the end of the study; $n = 8-9$ mice/group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.0001$, compared with indicated group.

Ketone Ester Supplementation Decreases the Methacholine Hyperresponsiveness of HFD-Induced Obese Mice

Ketone esters are a dietary supplement approved for human use that transiently increase circulating BHB levels (55). C57BL/6J mice were maintained on LFD or HFD until the HFD-fed mice were obese, and then mice were continued on the same food or a cohort of the obese mice were administered a HFD containing 20% by weight ketone ester (HFD + KE) for 3 wk. The body weight of the mice at the end of the feeding regimens was elevated in both the HFD and HFD + KE groups, although there was a trend toward a

decreased weight in the HFD + KE mice compared with the HFD mice that did not reach significance (Fig. 5A). Whereas serum BHB levels were slightly elevated in the HFD-fed mice compared with the LFD-fed mice, the levels were further substantially and significantly elevated in the HFD + KE mice (Fig. 5B). The BAL alveolar macrophage numbers were not different between the groups (Fig. 5C). The HFD-fed mice exhibited methacholine hyperresponsiveness, particularly in central airway resistance and tissue damping, whereas the HFD + KE mice exhibited significantly attenuated methacholine responsiveness in both parameters R_N and G (Fig. 5, D-F). Additionally, whereas serum 8-isoprostane levels were not different between the groups (Fig. 5G),

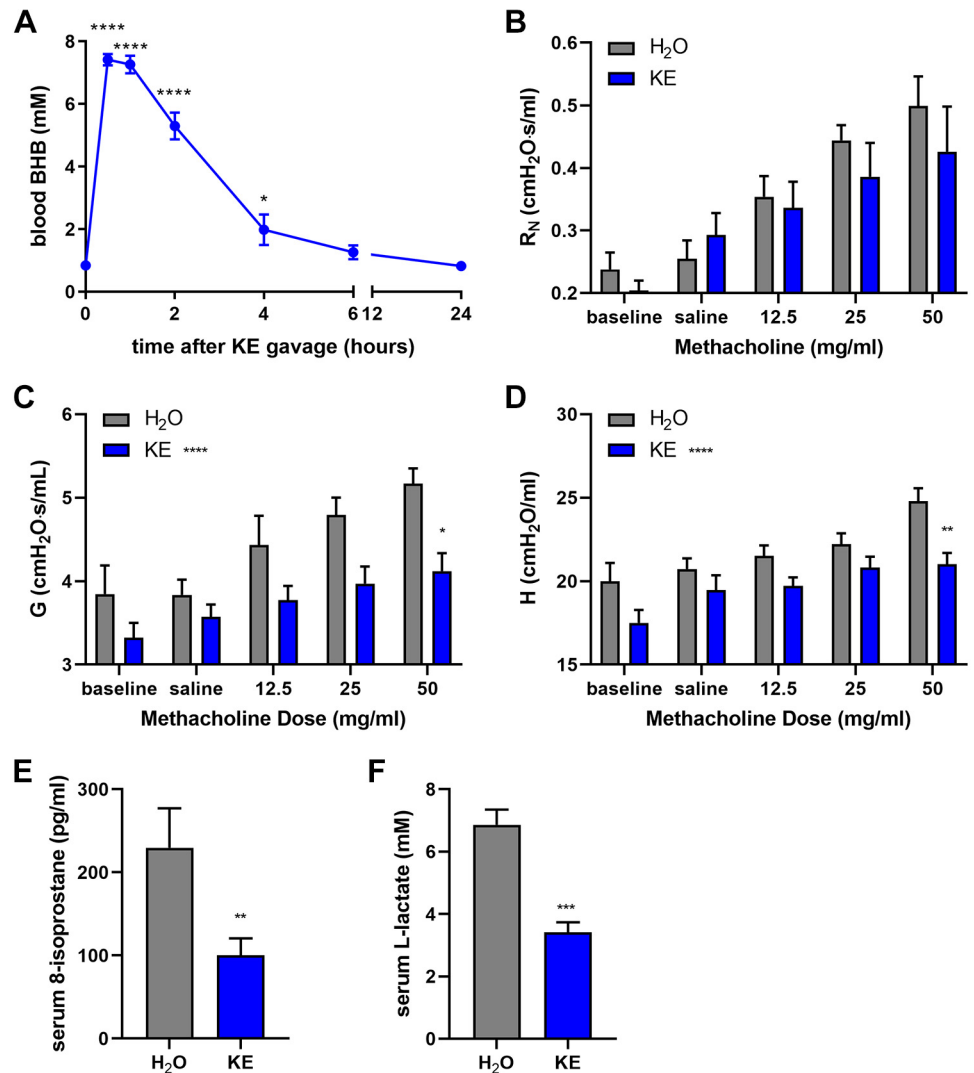


Figure 4. Ketone ester administration acutely decreases methacholine responsiveness. Mice were administered 200 μ L of water (H₂O) or ketone ester (KE; ~4 g/kg) by oral gavage. Blood β -hydroxybutyrate (BHB) (A) was measured before or at several time points following KE administration; $n = 5$ mice/group. Airway resistance (R_N) (B), tissue damping (G) (C), and tissue elastance (H) (D) were measured 1 h following water or ketone ester gavage. Serum 8-isoprostane (E) and lactate (F) were measured; $n = 6$ mice/group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, compared with time 0 by ANOVA (A) or the water group by t test (E and F). * $P \leq 0.05$, ** $P \leq 0.01$, compared with water group at the same dose of methacholine (B–D).

serum lactate levels in mice fed the HFD + KE were significantly decreased compared with HFD-fed mice (Fig. 5H). Finally, lung expression of several genes of potential relevance to the mechanism whereby ketone ester feeding may elicit an inhibitory effect on methacholine hyperresponsiveness was assessed. Lung expression of adipogenic (*Adipoq*), epithelial (*Muc5ac* and *Muc5b*), mesenchymal (*Col1a1* and *Actg*), muscle (*Myh11*, *Myod*, and *Myog*), and obesity-associated proinflammatory genes (*Ccl2*, *Tnfa*, *Il6*, *Il17a*, *Saa3*, and *Nlrp3*) was not significantly different between LFD and HFD, and none was not significantly different between HFD and HFD + KE (Fig. 5I). Expression of the epithelial gene chloride channel accessory 1 (*Clca1*), the smooth muscle and mesenchymal genes actin alpha 2 (*Acta2*), vimentin (*Vim*), and transgelin (*Tagln*), as well as the proinflammatory cytokine IL-1 β (*Il1b*) was significantly increased in the lungs of HFD-fed mice compared with LFD-fed mice. However, expression of these genes was not significantly decreased in the HFD + KE lungs compared with those from HFD-fed mice (Fig. 5I). Expression in the lung of tight junction protein 1 (*Tjp1*) was significantly elevated in the HFD + KE group compared with the HFD group (Fig. 5I).

Dietary Ketone Ester Supplementation Transiently Promotes an Antiobesogenic Gut Microbiome

We have previously reported that diet-induced weight loss in obese mice previously fed a high-fat diet promotes an antiobesogenic gut microbiome characterized by decreases in the elevated fecal *Firmicutes/Bacteroidetes* ratio present in obesity (18). To examine the impact ketone ester supplementation has on the fecal microbiome, normal chow-fed C57BL/6 mice were fed normal chow containing 20% water or KE (by weight) for 15 days then fed normal chow for an additional 15 days. Whereas mice fed the normal chow (even with 20% water) increased body weight steadily over the course of the study, mice fed chow containing KE displayed a transient decrease in body weight followed by a similar slope of body weight increase after day 5 of the KE feeding and throughout the remainder of the study (Fig. 6A). Whereas there were no differences in the overall abundance of total eubacterial 16S rRNA in the feces (Fig. 6B), there were significantly elevated abundances of *Bacteroidetes* in the KE-fed mice at 15 days that

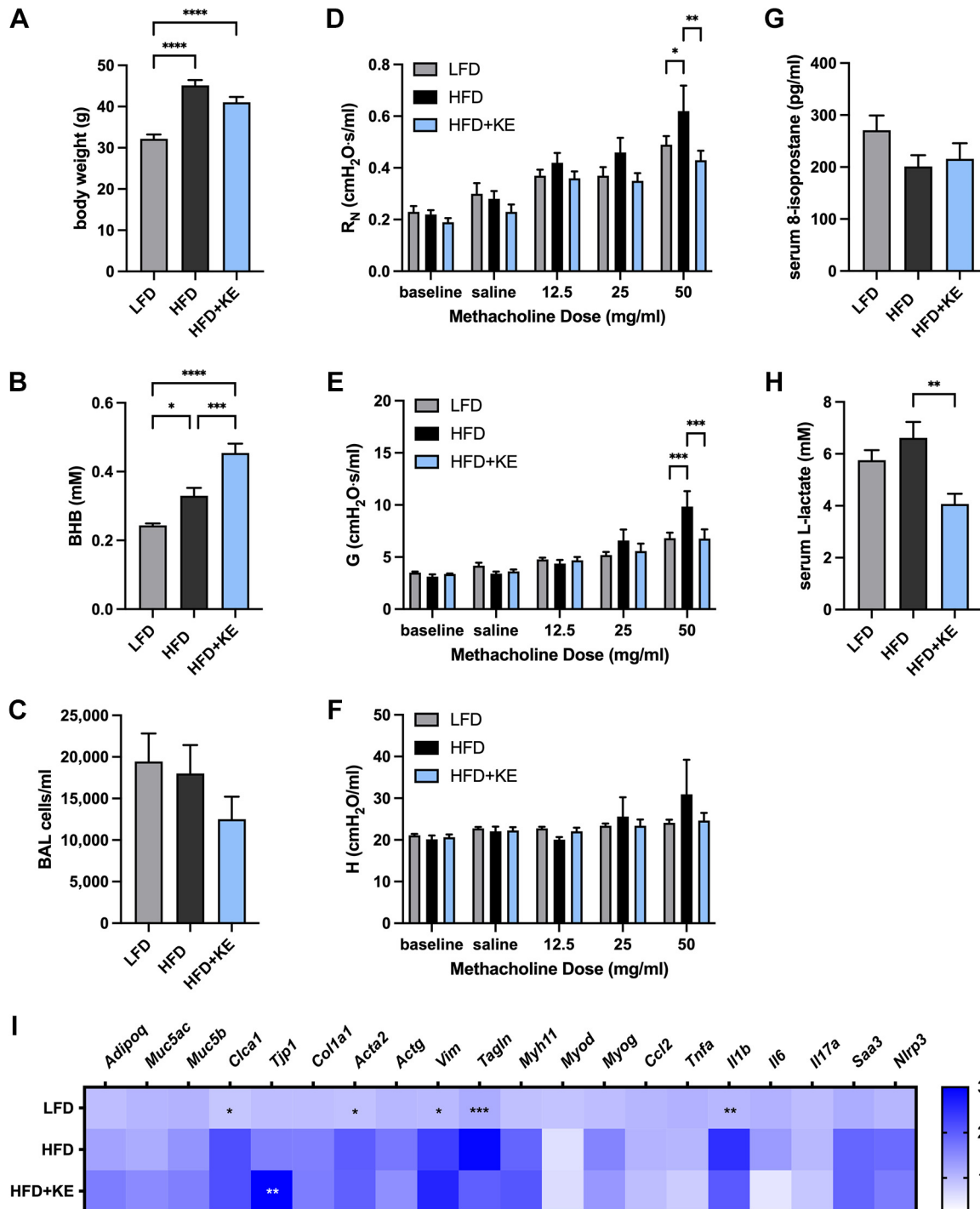


Figure 5. Ketone ester supplementation decreases methacholine hyperresponsiveness in high-fat diet-induced obese mice. Mice were maintained on a low-fat diet (LFD), a high-fat diet (HFD), or a high-fat diet containing 20% (by weight) ketone ester (HFD + KE) for 3 wk. Body weight (A), serum β -hydroxybutyrate (BHB) (B), and total bronchoalveolar lavage (BAL) cells (C) were measured. Airway resistance (R_N) (D), tissue damping (G) (E), and tissue elastance (H) (F) were measured. Serum 8-isoprostane (G), serum lactate (H), and lung gene expression were measured and presented as expression relative to the mean of the LFD group, scaling from 0 to 3 (I); $n = 8$ –10 mice/group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, compared with indicated group (A–H) or HFD group (I).

remained following an additional 15 days of feeding normal chow (Fig. 6C). In mice fed KE for 15 days then fed normal chow, fecal *Firmicutes* levels were significantly elevated (Fig. 6D), whereas there were no differences in the abundance of *Proteobacteria* between the groups

(Fig. 6E). At the end of the first 15-day KE-feeding period, the calculated fecal *Firmicutes/Bacteroidetes* ratio was substantially decreased relative to the chow-fed mice, an effect that was no longer present on day 30, after 15 days of again feeding normal chow (Fig. 6F).

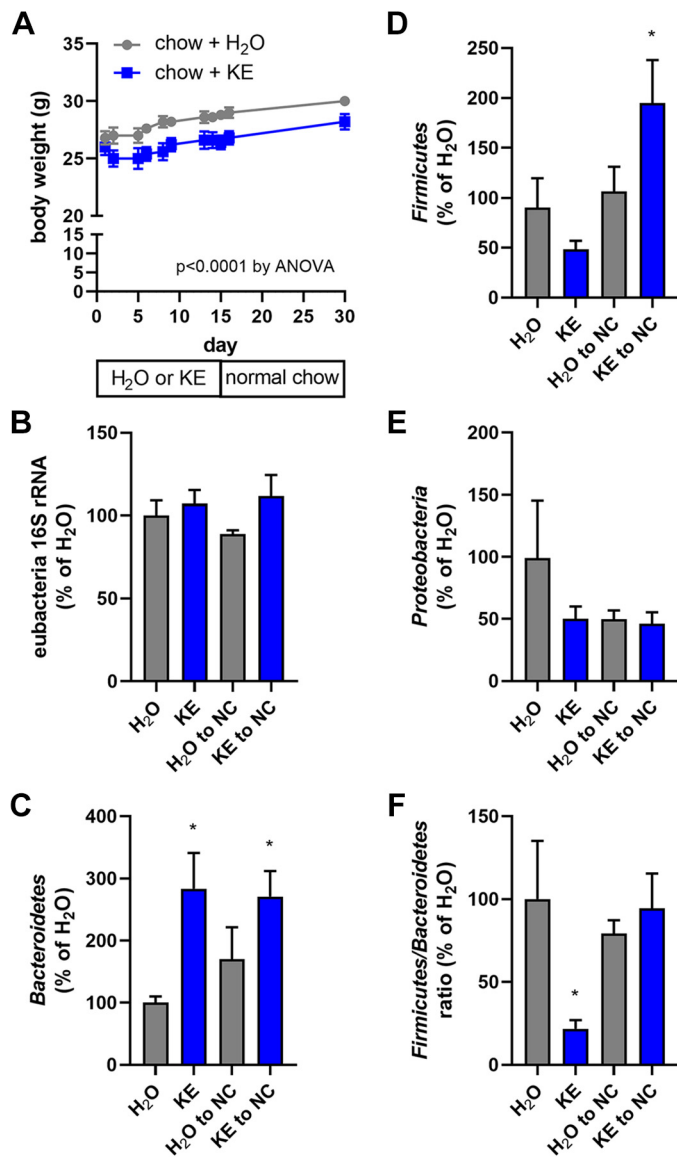


Figure 6. Ketone ester feeding promotes a transient anti-obesogenic gut microbiome. Mice were maintained on normal chow until day 0 and then maintained on chow containing 20% (by weight) water (H₂O) or 20% ketone ester (KE) for 15 days and then switched back to normal chow (NC) until day 30. Body weight was measured on several occasions throughout the 30-day feeding regimen (A). Fecal pellet total eubacterial 16S rRNA (B) and phyla-specific 16S rRNA for *Bacteroidetes* (C), *Firmicutes* (D), and *Proteobacteria* (E) DNA content was quantitated on days 15 and 30, and expressed relative to that found in the chow + H₂O fed mice at day 15. The *Firmicutes/Bacteroidetes* ratio, relative to that found in the chow + H₂O fed mice at day 15, was calculated (F); $n = 5$ mice/group. * $P \leq 0.05$, compared with indicated group.

DISCUSSION

Given the rapidly worsening global epidemics of asthma (82) and obesity (42), and the heterogeneity of these conditions (83, 84), there is a pressing need to understand the efficacy of and mechanisms through which mediators associated with the beneficial effects of weight loss, which may provide novel insight into the treatment of obese asthma not afforded by conventional approaches. Our studies demonstrate that dietary treat-

ments inducing acute (ketone ester gavage), intermediate duration (low-fat diet-induced weight loss), or protracted (ketogenic diet or ketone ester supplementation) increases in the ketone body β -hydroxybutyrate decrease methacholine responsiveness and obesity-associated methacholine hyperresponsiveness. Whereas the physiological mechanisms underlying the methacholine hyperresponsiveness in obese asthma are uncertain and remain the subject of investigation (85), both large airway and peripheral lung dysfunction are present in obese asthma (15–17), and our interventions show that increased ketone body concentrations benefit both the proximal and distal airways. First, ketone bodies decreased the parameter R_N that provides a measure of the flow resistance of the entire airway tree. R_N is more sensitive to contraction of the central rather than the peripheral airways (86). Second, the interventions also decreased tissue damping, G , and tissue elastance, H . Both G and H are increased by the development of heterogeneous ventilation to the distal reaches of the lung due to variations in airway narrowing, and also by derecruitment of lung units (86). Both ventilation heterogeneity and derecruitment are events that manifest predominately in the periphery of the lung and that are particularly sensitive to contraction of peripheral airways (87). However, the central airways and the lung periphery are not mechanically independent because the parenchymal tissue is tethered to the airways (85). It is therefore compelling to speculate that a primary effect of ketones may be on bronchial smooth muscle cells, attenuating their capacity to contract in response to methacholine and thereby affecting the changes in all three mechanics parameters: R_N , G , and H . Based on the results reported herein, we posit that providing elevated levels of ketone bodies in the setting of obesity could provide benefit (53) to intrinsic asthma and may do so through a number of direct and indirect effects on lung physiology throughout the proximal airways and distal airspaces that modulate inhaled methacholine hyperresponsiveness.

Intriguingly, in obese subjects undergoing bariatric surgery, ketone body levels in the circulation rise very rapidly, even before substantial weight loss is realized (88), coinciding with relief from many asthma-associated comorbidities. Similarly, our high-fat diet to low-fat diet weight loss model induced rapid and transient elevations, over the course of 6 days, in systemic ketone body concentrations that preceded and accompanied substantial weight loss. Healthy weight loss induces the mobilization of fat from adipose tissue that is subsequently catabolized in the liver through β -oxidation to form the ketone bodies β -hydroxybutyrate (BHB) and acetoacetate (AcAc), which then enter the circulation and can be utilized by cells throughout the body. Ketone bodies exert their metabolic functions within mitochondria through their entry into the tricarboxylic acid cycle to generate 1 GTP and 11 ATP molecules per acetyl group oxidized (77). Ketones also decrease circulating levels of glucose and the rate of glycolysis, even in states where carbohydrates are abundant (89–91). As an increased rate of glycolysis can promote inflammation (92, 93), it is possible that some of the beneficial effects of ketones observed in our studies are derived from their ability to provide an alternative fuel source to glucose and promote oxidative phosphorylation. The

consequences would be a decreased production of lactate, which has been implicated as a pathogenic driver of asthma (79–81).

In our acute studies of ketone ester supplementation that decreased methacholine-induced tissue damping and tissue elastance, circulating lactate concentrations were significantly decreased. In addition, compared with mice fed a high-fat diet, the fed a high-fat diet-fed mice supplemented with ketone ester had lower levels of serum lactate. These results show less utilization of glucose as a cellular fuel when ketone body concentrations are elevated. Ketone bodies also perform additional functions systemically in concert with and independent of their ability to function as a cellular fuel (50, 51, 61). Ketone bodies have redox-modulating and anti-inflammatory functions. Airway mitochondria are altered in animals fed a high-fat diet (32), and mitochondrial reactive oxygen species are involved in the complications of obesity (29, 33, 94). Furthermore, increased transcription and release of cytokines (95, 96) are a consequence of signaling through redox-sensitive pathways (97, 98). Ketones decrease ROS production by acting directly as antioxidants and by inducing the expression of genes associated with resistance to oxidative stress (54, 55, 75, 76). Increased methacholine reactivity is observed in allergen-challenged obese mice (99–102) and elevated levels of oxidative stress in the airways of obese asthmatics increase proportionally with BMI (13). Acute ketone ester supplementation significantly decreased circulating levels of 8-isoprostanes, a biomarker of lipid peroxidation elevated in obesity (103). In overweight asthmatics, the improved clinical findings and decreases in oxidative stress in subjects practicing alternate day calorie restriction are associated with elevated circulating levels of ketone bodies (BHB) (64).

In contrast to their association with ketoacidosis, a pathogenic state that can come about in the setting of diabetes and alcohol consumption (104) in which circulating ketone levels are well above those achieved during the feeding of a ketogenic diet or starvation (51), elevating ketone bodies is safe both in animal models of disease and in human subjects (65, 90, 91, 105–110). A ketogenic diet contains sufficient protein, reduced carbohydrates, and an abundance of fat that serves as a substrate for ketone body formation (71). In our hands, feeding a ketogenic diet for 10 days to mice previously fed a high-fat diet augmented circulating BHB levels but not to remarkably high concentrations. Other methods to rapidly increase the levels of circulating ketone bodies include the feeding of ketone esters that provide long-lived and high levels of ketone bodies. Ketone esters are generally regarded as safe (meaning they can be considered a dietary supplement) and have shown benefits to elite athletes and in patients with chronic disease (65, 90, 91, 105, 106). As used in our studies, the ketone ester (R)-1,3-beta hydroxybutyrate (R)-1,3-butanediol (91, 105, 106) augments circulating BHB levels and was provided by gavage akin to how it is acutely consumed as a supplement, or incorporated into mouse food at 20% of weight (and approximately calories) to promote protracted consumption. This ketone ester supplementation strategy could be optimized as an approach to promote a state of “therapeutic ketosis” similar to that achieved through the feeding of a ketogenic diet or fasting,

without any caloric deficit or the need for substantial lifestyle modification.

Ketone bodies can also function through the cell surface receptors hydroxycarboxylic acid receptor 2 (HCAR2/GPR109a) and free fatty acid receptor 3 (FFAR3/GPR41) (50, 51, 111, 112). Additionally, ketone bodies have also been reported to function as class-I histone deacetylase (HDAC) inhibitors (50, 51, 76), and to induce β -hydroxybutyrylation of histone H3 lysines (113) to influence gene expression. We detected increased expression of the smooth muscle-associated genes, *Tagln* (transgelin/SM-22 alpha) and *Acta2* (actin alpha 2), in the lungs of methacholine-hyperresponsive, HFD-fed obese mice, which appeared to be decreased in obese mice fed HFD containing ketone ester. *Acta2* encodes alpha smooth muscle actin, necessary for agonist-induced contraction and elevated in asthmatics (114). *Tagln* encodes an actin-crosslinking protein, which although not affecting the rate of actin filament propulsion is also elevated in asthmatic airways (114). In mice fed high-fat diet supplemented with ketone ester, lung expression of the tight junction protein *Tjp1* was significantly increased, which may augment airway barrier integrity and limit access of inhaled methacholine to the underlying smooth muscle, thereby decreasing responsiveness. It is possible that BHB affects bronchial smooth muscle responses to methacholine, which could inhibit contraction at many distinct cellular levels. Expression of the gene encoding IL-1 β was significantly elevated in the lungs of high-fat diet-fed mice and was not as highly expressed in obese mice provided ketone ester supplementation. BHB inhibits NLRP3 inflammasome-mediated IL-1 β processing (56, 62), and coupled with decreased lung *Il1b* expression could translate into substantially decreased bioactive IL-1 β , a cytokine that is a cause of elevated glycolysis and accompanying pathology in asthma (80, 115, 116).

Our results provide innovative first steps toward a safe, efficacious, and cost-effective strategy that may uniquely target obese asthma. However, these results may also be translatable to allergic asthma in which a more robust inflammatory response is present. While it is widely-accepted that 5–10% weight loss is required to achieve improvement in obese asthmatics (46), the modest reductions in weight elicited by feeding a ketogenic diet or providing ketone ester supplementation implicate effects besides weight loss for which BHB may be responsible. While a transient “antiobesogenic” gut microbiota was achieved following dietary supplementation with the ketone ester, weight gains over time were similar between the treated and untreated groups. The decreased *Firmicutes/Bacteroidetes* ratio present during ketone ester supplementation may also provide short-chain fatty acids, such as butyrate, which has been reported to elicit therapeutic effects in asthma (117). Butyrate may even be a substrate for conversion into BHB endogenously (118), suggesting that some of the beneficial effects of butyrate may be due to BHB. Intriguingly, medium-chain triglyceride supplementation is a therapeutic arm of The PrecISE Network, a National Heart, Lung, and Blood Institute-sponsored trial providing biomarker-based investigational interventions to patients with specific endotypes of severe asthma (119). Medium-chain triglycerides are preferentially catabolized to form ketone bodies, and BHB levels are a target, although not necessarily a mediator, for

achieving potentially therapeutic levels of supplementation in this intervention arm targeted at high-arginine metabolic severe asthma (119). Certainly, metabolic therapy for obese asthma is a potential complement or alternative to other conventional approaches for the treatment of these patients.

DATA AVAILABILITY

The data that support this study are available from the author on reasonable request.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.M.M. and M.E.P. conceived and designed research; M.M.M., L.F.R., C.J.W., M.L.T.B., J.L.A., and M.E.P. performed experiments; M.M.M., J.L.A., and M.E.P. analyzed data; M.M.M. and M.E.P. interpreted results of experiments; M.E.P. prepared figures; M.E.P. drafted manuscript; M.M.M., L.F.R., C.J.W., M.L.T.B., and M.E.P. edited and revised manuscript; M.M.M., L.F.R., C.J.W., M.L.T.B., J.L.A., and M.E.P. approved final version of manuscript.

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