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Materials and Methods

Animals

All animal protocols were approved by the Yale University School of Medicine Institutional Animal Care and Use Committee. Normal male Sprague-Dawley rats (~350 g, ~12 weeks of age) were ordered from Charles River and allowed to acclimate for 1 week prior to any studies or surgical procedures. The animals were assigned to treatment groups ensuring that the average body weight of the groups was matched at the beginning of each study, with at least one rat per cage assigned to each treatment group. For practical reasons, investigators were not blinded as to the group allocation. The rats underwent surgery under general anesthesia with isoflurane to place polyethylene catheters in the right jugular vein and the left carotid artery (PE90 and PE50 tubing, respectively). In addition, rats undergoing chronic intragastric infusion or fecal transplant studies had a third polyethylene catheter (PE50 tubing) placed in the antrum of the stomach, rats given ICV acetate had a catheter placed in the third ventricle of the brain, rats treated with NTS acetate had bilateral catheters placed in the nuclei tracti solitarii, and in rats used for the vagotomy studies, the right cervical vagus nerve was ligated in the carotid sheath. Rats were monitored daily for 3 days after surgery, with the exception of vagotomized rats, which were monitored for 7 days after surgery. Rats would have been euthanized had they lost more than 15% of their pre-surgery body weight or exhibited vomiting, diarrhea, lethargy, tachycardia, or tachypnea; however, none of these adverse effects occurred. Unless otherwise specified, rats were fasted for 12 hours (overnight) prior to any clamp or tracer infusion study.

In the portal vein ligation studies, rats were placed under isoflurane anesthesia and infused with tracer (2 μmol/(kg-min)) acetate. A blood sample (300 μl) was taken for measurement of acetate enrichment at the end of the infusion period, and the region of interest, below the splenic juncture, was ligated with simple sutures with the acetate tracer continuing. 20 min after ligation of the region of interest, a second blood sample was taken for measurement of acetate enrichment, and the animal was euthanized.

In the acute colonectomy studies, rats were placed under anesthesia and acetate turnover was measured as described above. A suture was placed encompassing the left and right side of the colon (around the cecocolic junction and the rectum) and the segment of the intestines between these sutures was removed. The tracer infusion was continued for an additional 20 min, and acetate turnover was measured and the animal euthanized as described above.

In the colon washout studies, rats were placed under anesthesia and acetate turnover was measured as described above. A clamp was then placed at the cecocolic juncture to avoid backflow from large to small intestine, and 30 mL normal saline were injected into the proximal colon. The outflow from the anus was collected, and the acetate tracer infusion was continued for 20 min, with acetate turnover measured and the animal euthanized as described above.

Germ-free (GF) Swiss Webster mice (mixed sex, 25-27 weeks of age) were maintained in flexible plastic isolators and were fed irradiated diet (chow, TD.140806 or high fat diet, TD.06414) for 4 weeks. GF status was verified by 16S rRNA-targeted PCR and anaerobic and aerobic culturing. Conventionalized (CONV-D) mice were generated by inoculating GF mice by oral gavage with chow fed mouse feces prepared under anaerobic conditions in anaerobic PBS and housing for 1 week in dirty bedding from chow fed mice. On the day of inoculation, they were started on chow (TD.140806) or HFD (TD.06414) and maintained on those diets for 4 weeks. After the 4 week period, all mice were sacrificed using isoflurane anesthesia and plasma and tissues were isolated.

Dietary interventions

Rats were fed one of the following diets for the time periods specified in the text.

Diet	Vendor, Product #	Feeding period	% Carbo-hydrate calories	% Fat calories	% Protein calories
High carbohydrate	Harlan 2018	1 week (pair feeding), 4 weeks (all other studies)	58	18	24
High fat	Dyets 112245	1 week (pair feeding), 3 days, 4 weeks	26	59	15
Very high fat	VWR 89408-206	1 week	2	93	5

Rats fed the high fat-high carbohydrate diet were given *ad lib* access to the high fat diet listed above as well as *ad lib* access to 5% sucrose drinking water for 1 week. Food intake was measured by weighing the food (and, if applicable, sucrose water) given at the beginning of a study and the weight of food remaining each day and multiplying the food consumed by the caloric content per gram of food.

Rats used for the bicarbonate feeding studies were given ^{13}C sodium bicarbonate in drinking water (300 mg/L) and in food (1 mg/g). The chow pellets were ground before mixing in the sodium bicarbonate. Rats were fed ^{13}C bicarbonate labeled food for three days before they were sacrificed and tissues isolated. GF and CONV-D mice used for the bicarbonate feeding studies were given ^{13}C sodium bicarbonate in drinking water (300 mg/L) only for 3 days. The water administered to both the GF and CONV-D mice was autoclaved prior to being given to the animals.

In the fasting study, a group of 4 week high fat fed rats was fasted for 48 hours prior to a hyperglycemic clamp and acetate tracer (2 μmol/(kg-min)) study. At the conclusion of this study, they immediately underwent a second hyperglycemic clamp study with replacement doses of acetate (20 μmol/(kg-min)). These rats were compared to a unique group of 4 week high fat fed rats fasted for 12 hours prior to a hyperglycemic clamp and acetate tracer study.

Hyperglycemic clamps

Rats were infused with varying doses of 20% glucose to maintain plasma glucose ~160-170 mg/dL through an inter-arterial catheter. Blood samples (200 μL whole blood) were taken at time 0, 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min of the clamp for measurement of plasma glucose and insulin concentrations. At 120 min, rats were sacrificed by administration of intravenous sodium pentobarbital and livers were isolated and flash frozen in liquid N_2. Tissues and plasma were stored at -80 C for further analysis.

Euglycemic clamps

Overnight fasted rats undergoing the hyperinsulinemic-euglycemic clamp were given a 40 mU/kg bolus of Regular insulin at time 0 of the clamp, immediately followed by intra-arterial infusion of Regular insulin (4 mU/[kg-min]) and variable ~20% dextrose (2.5% [6,6-$^2H_2$]glucose) to maintain euglcyemia (100-110 mg/dl). Blood samples were taken every 15 min during the clamp through a catheter in the jugular vein, and glucose infusion rates adjusted as necessary. At 120 min of the clamp, the rats were euthanized by IV pentobarbital. Plasma [6,6-$^2H_2$]glucose enrichment at steady state was measured by GC/MS in the EI mode, and the glucose disposal rate was calculated as

$$Glucose\ disposal\ rate = \frac{Tracer\ enrichment}{Plasma\ enrichment} * Glucose\ infusion\ rate$$

with endogenous glucose production calculated as

$$Endogenous\ glucose\ production = Glucose\ disposal\ rate - Glucose\ infusion\ rate.$$

Glucose tolerance tests

In the glucose tolerance tests, rats were given an intraperitoneal injection of 1 g/kg glucose at time 0. Blood samples (200 µL whole blood) were taken 0, 15, 30, 45, 60, 90, 120, and 180 min after the glucose injection for measurement of plasma glucose and insulin concentrations and rats were sacrificed at 180 min using pentobarbital as described above.

Acetate, propionate, and butyrate turnover studies

Rats used for the acetate turnover studies were given a primed-continuous infusion of $^{13}C_1$ sodium acetate (350 mM) at the following infusion rates through an intra-arterial catheter during the hyperglycemic clamp study:

Dose	Prime 1 (0-5 min)	Prime 2 (5-10 min)	Continuous (10-120 min)
Tracer (2 µmol/(kg-min)	5.5 µmol/(kg-min)	2.8 µmol/(kg-min)	2.0 µmol/(kg-min)
8 µmol/(kg-min)	22 µmol/(kg-min)	11 µmol/(kg-min)	8.0 µmol/(kg-min)
20 µmol/(kg-min)	55 µmol/(kg-min)	28 µmol/(kg-min)	20 µmol/(kg-min)

Unless otherwise specified in the text, all acetate turnover studies used a continuous infusion rate of 2.0 µmol/(kg-min). Blood samples (200 µL whole blood) were taken at 120 min for measurement of plasma acetate enrichment as described below. Rats were then sacrificed using sodium pentobarbital and tissues isolated as described above.

For the propionate and butyrate tracer studies, rats were co-infused with U-^{13}C sodium propionate and $^{13}C_1$ sodium butyrate at a rate of 0.1 µmol/(kg-min) for each tracer during a hyperglycemic clamp. Blood samples were taken and rats sacrificed as described above.

For the butyrate replacement study, rats were infused with 0.5 µmol/(kg-min) $^{13}C_1$ sodium butyrate during a hyperglycemic clamp performed as described above. These rats were compared to a unique group of rats given tracer doses (0.1 µmol/(kg-min)) of $^{13}C_1$ sodium butyrate.

ICV acetate administration

Rats with catheters in the carotid artery, jugular vein, and third ventricle of the brain were treated with 0.96 µg acetate in 20 µL sterile phosphate buffered saline (pH 7.42), or saline control using the intracerebroventricular catheter. Immediately following the acetate or saline injection, they underwent a hyperglycemic clamp study as described above.

NTS acetate administration

Rats with catheters in the carotid artery, jugular vein, and bilateral nuclei tracti solitarii were treated with 0.96 µg acetate in 5 µL sterile phosphate buffered saline or saline control. They then went hyperglycemic clamps as described above.

Chronic intragastric acetate infusion

Ad lib chow fed rats were infused with acetate (20 µmol/(kg-min)) through an intragastric catheter continuously for ten days. A polyethylene harness (Instech) was used to protect the catheters. Body weight was measured on days 0 and 10 of infusion. Food intake was measured by weighing the food available to the rat on days 0 and 10.

GLP-1 inhibition

In the GLP-1 inhibitor studies, rats were treated with a competitive inhibitor of GLP-1 activity, exendin fragment 9-39 (Sigma) (5 µg/kg) in sterile saline (or saline control) by intraperitoneal injection 30 min prior to the start of a hyperglycemic clamp and acetate infusion (20 µmol/(kg-min)) study using the protocol described above.

Atropine and methylatropine treatment

Rats infused with 20 µmol/(kg-min) intra-arterial acetate or given 0.96 µg ICV acetate were treated with 0.1 mg/kg intravenous atropine (Sigma) in sterile saline (or saline control) 15 min before the start of a hyperglycemic clamp performed as described above. In the ICV methylatropine treatment studies, rats received 30.4 ng methylatropine in 10 µl sterile saline 15 min prior to the initiation of a hyperglycemic clamp with infusion of 20 µmol/(kg-min) intra-arterial acetate.

Antibiotics treatment

In the antibiotics treatment studies, 4 week high fat fed rats were treated with a seven-day course of oral vancomycin (25 mg/kg per day), gentamycin (20 mg/kg per day), and rifampin (5 mg/kg per day) (Sigma). The day after the last dose of antibiotics, they underwent acetate tracer and hyperglycemic clamp studies as described above.

Fecal transplantation

Fecal transplants were performed once daily for 3 days. Chow fed rats were transplanted with chow fed rat feces, chow fed rats were transplanted with 4 week HFD rat feces, and 4 week HFD rats were transplanted with chow fed rat feces. Rats from independent litters were randomized across treatment groups prior to diet administration or fecal transplantation. For the fecal transplantation studies, anaerobic transport tubes were prepared by storing cryo-tubes in an anaerobic chamber for >24h prior to use, and then adding 1mL anaerobic PBS-cysteine inside the anaerobic chamber. Tubes were sealed and opened for no more than 1-2 s for collection of freshly voided fecal pellets (3/animal). The sealed tubes were vortexed for 30 s prior to injection into recipient animals through an intragastric catheter. To confirm anaerobic conditions, matched tubes of PBS-cysteine containing the oxygen indicator Resazurin (1 mg/L) were subjected to identical treatment; no color change was observed. Resazurin was not added to the samples used for fecal transplantation because the feces color would mask the color change of the indicator.

16S sequencing

Culture-independent microbiome profiling was performed as described.[1,2] Briefly, fecal samples were homogenized by bead-beating (BioSpec Inc.), phenol-chloroform extraction, and column purification and the V4 region of the 16S rRNA gene was PCR-amplified using dual barcoded primers.[3] Amplicons were pooled and sequenced on an Illumina MiSeq instrument using paired-end, 2x250bp reads. Paired end reads were assembled as described[2] and assigned to samples based on the dual barcodes using a q20 cutoff in QIIME v1.7.[4] Reads were assigned to operational taxonomic units at a 97% identity cutoff using open reference clustering and taxonomic assignments were generated using the gg_13_5 reference database.[5] Samples were subsampled to 10,000 reads per sample and singletons removed prior to beta diversity analysis in QIIME v1.7. Distance matrices based on Bray-Curtis, Hellinger, unweighted UniFrac, and weighted UniFrac distances produced similar results.

Measurement of short-chain fatty acid concentrations and turnover

Plasma acetate, propionate, and butyrate concentration and enrichment were measured after 120 min of tracer infusion by GC/MS. 30 µL plasma were spiked with an equal volume of d_4 sodium acetate (0.5 mM) and acidified with 50 µL 1 M HCl. After shaking for 20 min, samples were derivatized with 40 µmol 1,3-dicyclohexylcarlodiimide and 40 µmol 2,4-difluoroaniline, shaken for 1 hour, and dried under N_2 gas. Samples were then resuspended in ethyl acetate

and peak areas (mass to charge ratios: unlabeled acetate, 171; $^{13}C_1$ acetate, 172; d_4 acetate, 174; unlabeled propionate, 185; U-$^{13}C_3$ propionate, 188; unlabeled butyrate, 199; $^{13}C_1$ butyrate, 200) measured by gas chromatography/mass spectrometry (Hewlett Packard). Whole-body turnover of each short-chain fatty acid was calculated using the formula

$$Turnover = \left(\frac{Tracer\ enrichment}{Plasma\ enrichment} - 1\right) * Tracer\ infusion\ rate$$

Concentrations were measured by comparing the peak area of each short chain fatty acid of interest to that of the internal standard (d_4 acetate).

In order to measure fecal and tissue short chain fatty acids normalized to wet weight, a piece of the sample (~100 mg) was weighed and fecal and tissue short chain fatty acid enrichment and concentration were measured using the extraction method of Turnbaugh et al.[6] and the GC/MS method described above. In order to normalize to dry weight while avoiding any methods that would volatilize the acetate, we suspended ~100 mg sample in 1 mL water and sonicated it for 30 sec. We then transferred this sample to a scintillation vial and dried it under N_2 gas. After drying down the sample for ~4 hr, we measured the weight of the dried-down tissue sample and measured short-chain fatty acid content in this sample as described above. Dietary acetate concentrations were measured by suspending ~100 mg of ground-up diet in 1 mL water and extracting and measuring acetate concentrations using the same method.

In vitro acetate production

Approximately 70 mg of feces was obtained from the ascending colon of high fat fed rats immediately following euthanasia with pentobarbital. The feces were incubated in simple media (DMEM without glucose), with or without [U-^{13}C] glucose or [U-^{13}C] palmitate added as specified in the text, at 37°C. Acetate concentrations were then measured in the incubation media by GC/MS as described above, with rates calculated assuming the concentration of acetate at time zero was 0, based on preliminary data.

Biochemical analysis

Plasma triglycerides were measured using an enzymatic method (Wako triglyceride assay), and tissue triglycerides were extracted using the method of Bligh and Dyer[7] and measured spectrophotometrically using an enzymatic reagent (Diagnostic Chemicals Ltd.). Tissue diacylglycerol concentrations were measured as we have described.[8] Plasma glucose was measured enzymatically using the YSI glucose analyzer. Rat plasma insulin, glucagon, gastrin, and total ghrelin were measured by ELISA (Mercodia, R&D Systems, Abcam, and Abnova, respectively), with mouse plasma gastrin and total ghrelin also measured by ELISA

(Sigma and Millipore, respectively). GLP-1 was measured using the MesoScale total GLP-1 (v2) kit. HOMA-IR was calculated using the formula

$$HOMA - IR = \frac{Glucose * Insulin}{405}.$$

Insulin area under the curve during the hyperglycemic clamps and glucose tolerance tests from time point a to subsequent time point b was calculated using the formula:

$$Insulin\ AUC = \frac{1}{2} * Time_{b-a} * (Insulin_a + Insulin_b)$$

and the total AUC was calculated by adding up all AUCs from time 0 to the end of the study. Plasma arginine, leucine, and alanine were measured by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) as we have described.[9] Plasma C2 acetylcarnitine concentrations were measured by LC/MS/MS as described by Griffin et al.[10]

Islet perifusion studies

Intact islets isolated from ~350g rats were hand picked and layered between acrylamide gel column beads (Bio-Gel P4G (156-4124)) either in perifusion media ((DMEM, Sigma D5030) supplemented with $NaHCO_3$ as per the manufacturer's instructions, 10 mM HEPES, 4 mM glutamine, 0.2% fatty acid free BSA and 2.5 mM glucose to simulate a physiologically relevant environment) or in KRB buffer within each perifusion column chamber. Eighty islets for each comparative condition (n=4) were perifused at a rate of 100 µl/min on an 8-channel BioRep Technologies perifusion device. Islets were equilibrated on the instrument in basal (2.5 mM) glucose perifusion media for 45 minutes prior to sample collection in a 5% CO_2/95% air, 37^{o}C constant environment. Either sodium acetate or acetylcarnitine was added acutely at the start of the perifusion collection sequence and was added continuously throughout the basal and glucose (9 mM) stimulation period. Rat insulin was measured in the perfusate using a high range rat insulin ELISA (ALPCO) and normalized to total islet DNA concentration using Quant-it PicoGreen dsDNA Assay Kit (Life Technologies).

Statistical analysis

GraphPad Prism was used to perform all statistical analysis including confirmation of similar variance between all groups compared. Significance was determined by the two-tailed unpaired Student's t-test when two groups were compared, or by one-way ANOVA with Bonferroni's multiple comparisons test when more than two groups were compared. P-values less than 0.05 were considered significant. In all cases, n=6 rats per group, with each data point representing a biological, not technical, replicate. The sample size was selected to detect

moderate-to-large (≥20%) differences between groups. All animals studied were included in the analysis.

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