Supplementary Data

Supplementary Materials and Methods

Plasma and liver triglycerides and total cholesterol and alanine aminotransferase levels

Plasma triglyceride and cholesterol concentrations were measured with enzymatic colorimetric assays from Wako Diagnostics (Richmond, VA), and alanine aminotransferase levels were measured with an enzymatic colorimetric assay from Bio Scientific (Austin, TX). Liver tissue was homogenized with sodium sulfate using a mortar and pestle, and lipids were extracted with a 2:1 chloroform–methanol mixture according to Folch et al.[1] A portion of the chloroform was evaporated under nitrogen gas and the sample was reconstituted with isopropanol and then assayed for triglyceride and cholesterol concentrations using enzymatic colorimetric assays from Fisher Diagnostics (Middletown, VA). These assays were conducted by the Mouse Metabolic Phenotyping Center at UC Davis.

Liver histology

Liver sections were stained with hematoxylin and eosin for histologic examination and the entirety of each liver section was evaluated in a blind manner by two pathologists. Inflammation most commonly consisted of neutrophils or lymphocytes in small clusters within the lobule and was graded on a five-point scale (grade 0, no inflammation; grade 1, minimal inflammation; grade 2, mild inflammation; grade 3, moderate inflammation; grade 4, severe inflammation). A single small cluster of neutrophils or lymphocytes within the entire liver cross section was considered sufficient for grade 1. Steatosis was most commonly present as a mixture of small- and large-droplet macrovesicular steatosis, and was graded on a five-point scale (grade 0, no steatosis; grade 1, minimal steatosis; grade 2, mild steatosis; grade 3, moderate steatosis; grade 4, severe steatosis). Fibrosis was graded on a five-point scale (grade 0, no fibrosis; grade 1, minimal lobular or portal fibrosis; grade 2, mild lobular or periportal fibrosis; grade 3, moderate lobular or bridging fibrosis; grade 4, severe lobular fibrosis or cirrhosis).

Hepatic iron

Given the known association between hepatic inflammation, steatosis, and iron accumulation,[2–4] we quantified hepatic iron. Tissue samples were analyzed at 10× dilution, using 18.2 MΩ-cm water by the Interdisciplinary Center for Plasma Mass Spectrometry at UC Davis using an Agilent 7500CE ICP-MS (Agilent Technologies, Palo Alto, CA).

Hepatic S*-adenosylmethionine and* S*-adenosylhomocysteine by HPLC*

Fifty milligrams of liver tissue was homogenized in 0.4 mL of cold 0.5 N perchloric acid and then centrifuged for 10 min at 14,000 rpm and 4°C. The supernatant was filtered through a 0.22-μm syringe filter and stored at –80°C until high performance liquid chromatography (HPLC) analysis. Sample preparation and analysis were performed within 4 weeks from tissue harvest to ensure sample stability.[5]

Global DNA methylation

DNA was isolated from C3H livers using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). The concentration and purity of the extracted DNA were determined by measuring absorbency at A260 and A280 on a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Relative methylation dot blots were performed according to an established method.[6] Fifty nanograms of genomic DNA was alkaline denatured and blotted onto a nitrocellulose membrane. After UV cross linking the DNA to the membrane, each blot was blocked with the Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) and incubated with the anti-5-methylcytosine primary antibody (Eurogentec, Fremont, CA) in blocking buffer +0.1% Tween-20 overnight at 4°C. Blots were washed in 1× PBS +0.1% Tween-20 and then incubated in blocking buffer +0.1% Tween-20 with the LI-Cor 700-IR secondary antibody (LI-Cor Biosciences). Blots were imaged using a LI-Cor Odyssey Imager (LI-Cor Biosciences, Lincoln, NE). Blots were rinsed using 2× SSC buffer before equilibration in the PerfectHyb Plus Hybridization Buffer (Sigma, St. Louis, MO) at 42°C. Biotin-labeled gDNA was hybridized to the membrane for 8 hr at 42°C. Blots were further incubated with the LI-Cor Streptavidin 800-IR secondary antibody (LI-Cor Biosciences) in blocking buffer +0.1% Tween-20 for 1 hr at room temperature and then imaged using the LI-Cor Odyssey Imager. Integrated intensities were quantified using the Odyssey software. Interassay variation of methylation signal was normalized to the same internal control sample in every assay.

Transcript levels of selected genes by qPCR

Total RNA was isolated from liver tissue using the RNeasy Mini Kit (Qiagen). Purity and concentration of extracted RNA were determined by NanoDrop spectrophotometry (Thermo Scientific). RNA integrity was determined through gel electrophoresis and samples were stored at –80°C until further analysis. Reverse transcriptase was performed using the SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA).

Primers were designed by the Real-Time PCR Research and Diagnostic Core Facility utilizing Primer Express 3 (Table 2). Primers were validated using 10-fold dilutions of a known positive control. Dilutions were analyzed in triplicate and a standard curve was plotted against the Cq of dilutions. The slope of the standard curve was used to calculate percent amplification efficiency using the formula $E = [10^{(1/-\text{slope})} - 1] \times 100$. A negative (water) control was included for each assay and the efficiencies for all assays were above 90%. Assay specificities were determined by NCBI nucleotide BLAST followed by post-qPCR melt curve analyses and visualization of qPCR products on an agarose gel.

SYBR green assays were used to detect transcript levels of selected genes; all samples were run in triplicate. Reaction conditions were 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Cycle threshold (Ct) values were normalized based on the housekeeping gene GADPH. The relative expression of a target gene was calculated using the equation $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta C_t$ (sample) us ΔC_t (calibrator).

Statistical analyses

Statistical analyses were performed to test effects of diet and sex using a one-way ANOVA. Where the overall ANOVA was significant, we performed a *post hoc* pairwise comparison to determine which diet groups differ. Tukey's method was applied to control for multiple comparisons. Spearman correlation coefficient and its *P* value for significance of correlation were calculated to assess the magnitude and direction of an association between two given variables. For data that were highly skewed, we applied a natural log transformation to achieve normality before statistical analysis and significance testing was done on a log-transformed scale. All reported *P* values are based on two-sided tests. A *P* value <0.05 was considered significant. All statistical analyses were performed using SAS, Version 9.4 (SAS Institute, Cary, NC).

References

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