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Dietary Betaine Supplementation Increases Fgf21 Levels to Improve Glucose Homeostasis and Reduce Hepatic Lipid Accumulation in Mice

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METABOLISM

Identifying markers of human insulin resistance may permit development of new approaches for treatment and prevention of type 2 diabetes. To this end, we analyzed the fasting plasma metabolome in metabolically characterized human volunteers across a spectrum of insulin resistance. We demonstrate that plasma betaine levels are reduced in insulin-resistant humans and correlate closely with insulin sensitivity. Moreover, betaine administration to mice with diet-induced obesity prevents the development of impaired glucose homeostasis, reduces hepatic lipid accumulation, increases white adipose oxidative capacity, and enhances whole-body energy expenditure. In parallel with these beneficial metabolic effects, betaine supplementation robustly increased hepatic and circulating fibroblast growth factor (Fgf)21 levels. Betaine administration failed to improve glucose homeostasis and liver fat content in *Fgf21*$^{-/-}$ mice, demonstrating that Fgf21 is necessary for betaine's beneficial effects. Together, these data indicate that dietary betaine increases Fgf21 levels to improve metabolic health in mice and suggest that betaine supplementation merits further investigation as a supplement for treatment or prevention of type 2 diabetes in humans.

Insulin resistance occurs years before the onset of type 2 diabetes and also predicts disease development (1). Fortunately, both intensive lifestyle programs (exercise, dietary modification) and pharmacotherapy are effective for type 2 diabetes prevention in insulin resistant humans (2). Thus, a key imperative is to identify individuals at highest risk to maximize success and cost-effectiveness of preventive strategies.

Advances in mass spectrometry–based metabolomics technology have permitted identification of novel metabolic signatures associated with risk for type 2 diabetes and other components of the metabolic syndrome (3–5). To identify biomarkers associated with insulin resistance and type 2 diabetes risk, we analyzed the fasting plasma metabolome in metabolically phenotyped human volunteers. We now report that insulin resistant humans have reduced plasma levels of N,N,N-trimethylglycine, or glycine betaine (hereafter referred to as betaine). Moreover, Walford et al. (6) report that plasma betaine levels not only are associated with reduced incidence of type 2 diabetes in a prospective cohort study but also predict successful response to prevention strategies.

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Betaine is a modified amino acid found in many foods, with particularly high content in whole grains (7). Both betaine intake and plasma levels are inversely correlated with several metabolic syndrome markers (8,9). Owing to its dipolar zwitterion structure, betaine functions as a key intracellular osmolyte. In mammals, betaine metabolism is dominant in the liver, where it serves as a methyl donor in the methionine cycle; the enzyme betaine-homocysteine methyltransferase catalyzes the reaction between betaine and homocysteine, yielding dimethylglycine and methionine (10). Betaine administration decreases homocysteine levels (11), a cardiovascular risk factor (12), and is used as treatment for homocysteinemia. Betaine decreases hepatic lipid content and improves glucose tolerance in rodents (13–16). However, the molecular mechanisms underlying these effects remain unknown.

Given the multiple lines of evidence supporting betaine regulation of metabolism, we tested the impact of betaine in mice with diet-induced obesity. We demonstrate that long-term betaine supplementation increases circulating levels of the systemic metabolic regulator fibroblast growth factor (Fgf)21, improves glucose homeostasis, reduces hepatic lipid, and increases whole-body energy expenditure. Betaine fails to improve metabolic health in mice lacking Fgf21, demonstrating that Fgf21 is required for the beneficial effects of betaine.

RESEARCH DESIGN AND METHODS

Human Metabolic and Metabolomics Analysis

Protocols were approved by the Joslin Diabetes Center Committee on Human Subjects, and informed consent was obtained from all participants. Plasma was obtained after an overnight fast from 40 healthy individuals with normal glucose tolerance. Insulin sensitivity was quantified by insulin-modified intravenous glucose tolerance with minimal-model analysis (17). Mass spectrometry (Metabolon, Inc.) was used to analyze 193 distinct metabolites. Plasma glucose was measured using glucose oxidase (2300 Stat Plus, YSI, Yellow Springs, OH) and insulin by radioimmunoassay (Diagnostic Systems Laboratories, Webster, TX).

Animal Experiments

All studies were approved by the Institutional Animal Care and Use Committee of Joslin Diabetes Center and the University of Barcelona. Male C57BL/6 mice (Harlan) were fed chow or a 45% or 60% kcal-from-fat diet (D12450B, D12451, and D12492, respectively; Research Diets) for 16 weeks. Betaine (Sigma-Aldrich) was administered in water (1% wt/vol) 1 week prior to starting high-fat feeding and was maintained throughout. *Fgf21*$^{-/-}$ mice (18) had been backcrossed with C57BL/6 mice for >10 generations and were treated using the same protocol and 45% fat diet.

Metabolic Analysis

Glucose (1.5 g/kg i.p.) and insulin (0.75 units/kg i.p.) tolerance tests were performed after overnight and 4-h fasts, respectively. Mice were fasted overnight and anesthetized prior to sacrifice. Hyperinsulinemic-euglycemic clamps were performed at the Mouse Metabolic Phenotyping Core, University of Massachusetts. After overnight fasting, a primed (150 mU/kg) continuous (15 pmol/kg/min) infusion of human insulin was initiated, together with 20% glucose at variable rates to maintain basal glucose. Insulin-stimulated glucose uptake was normalized to plasma insulin at the end of the 2-h clamp. Plasma insulin and Fgf21 were determined by ELISA (Crystal Chem and Millipore, respectively). Plasma betaine was quantified by liquid chromatography–mass spectrometry (LC-MS) after fractionation using an Acquity UPLC BEH HILIC column (Waters) and detection with a QqQ/MS 6490 mass spectrometer (Agilent). Betaine content of diets was quantified in methanol extracts using LC-MS (4000 QTRAP; AB Sciex). d9-betaine (C/D/N Isotopes) or $^{13}C_2$-betaine was used as internal standards for absolute quantification for plasma and chow, respectively. For 60% mouse high-fat diet studies, plasma and liver metabolites were analyzed by LC-MS (19). Liver betaine, S-adenosylmethionine, S-adenosylhomocysteine, methionine, and choline were quantified using stable-isotope dilution liquid chromatography–electrospray ionization tandem mass spectrometry (Baylor Research Institute, Dallas, TX) (20). Hepatic lipid was quantified in chloroform-methanol extracts (50 mg) using a Triglyceride Assay kit (Sigma-Aldrich).

Gene Expression

Total RNA was isolated with TRI Reagent (Sigma-Aldrich). cDNA was synthesized using a high-capacity cDNA kit (Applied Biosystems) and analyzed by real-time PCR using SYBR Green (Promega). Oligo sequences can be provided upon request.

Energy Expenditure

Mice were placed in individual chambers (Oxymax OPTO-M3 system; Columbus Instruments) with ad libitum access to food and water. After 24-h adaptation, O_2 consumption, CO_2 production, and activity were measured.

High-Resolution Respirometry

Mitochondrial function was assessed in freshly isolated tissue using high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) (21). Inguinal white adipose tissue (iWAT) was mechanically permeabilized (Shredder SG3, PBI Pressure BioSciences, Inc.) in respirometry medium (0.5 mmol/L EGTA, 3 mmol/L $MgCl_2$, 60 mmol/L K-lactobionate, 20 mmol/L taurine, 10 mmol/L KH_2PO_4, 20 mmol/L HEPES, 110 mmol/L sucrose, and 0.1% (wt/vol) BSA, pH 7.1). Oxygen flux was measured by adding malate (final 2 mmol/L), glutamate (10 mmol/L), and ADP (5 mmol/L) for complex I (*CI*) or succinate (10 mmol/L) for combined *CI+II* activity. Subsequently, oligomycin A (2 μg/mL) was added to inhibit ATP synthase (leak-dependent respiration). Finally, electron transport–independent flux was assessed after sequential addition of rotenone (0.1 μmol/L) and anti–mycin A (2.4 μmol/L)

and subtracted from values of each previous step. Values are expressed relative to protein.

Histological Analysis

Tissues were fixed, paraffin embedded, and stained with hematoxylin-eosin or Oil Red O (liver). For the pancreas, three nonconsecutive sections were labeled with anti-insulin (Dako) and anti-glucagon IgG (Sigma-Aldrich) and detected with Cy3- and Cy2-conjugated secondary antibodies (Jackson ImmunoResearch). Area of islets and adipocytes was measured in 30 images from three sections per animal using ImageJ by a blinded investigator.

Primary Hepatocytes

Primary hepatocytes were isolated from C57BL/6 mice after liver perfusion with collagenase and cultured overnight in gelatin-coated plates containing DMEM (Sigma-Aldrich), 10% FBS, and 1 μmol/L insulin and subsequently incubated with betaine.

Statistical Analysis

All data are presented as mean ± SEM. Between-group differences were analyzed using GraphPad Prism, using two-tailed *t* test or one-way ANOVA and a post hoc Tukey honestly significant difference test. Where stated, data were analyzed by two-way ANOVA with a post hoc Holm-Sidak test. Metabolomic data were log transformed and analyzed using R software (www.r-project.org); association with log insulin sensitivity index (S_I) was tested using Pearson correlation. *P* values were corrected for multiple comparisons using false discovery rate (www.jstor.org/stable/2346101). $P < 0.05$ was considered significant for all analyses.

RESULTS

Plasma Betaine Is Associated With Insulin Sensitivity in Humans

The plasma metabolome was analyzed in the fasting state from individuals across a spectrum of insulin sensitivity in order to evaluate the impact of insulin resistance. All participants underwent intravenous glucose tolerance tests to quantify insulin sensitivity (S_I) (22) (range 0.49–14.28). Participants were categorized as insulin sensitive (IS) or resistant (IR) based on S_I values above or below the median value of 4.78 for a larger population of normoglycemic individuals studied at the Joslin Diabetes Center (17). IR individuals had higher fasting plasma glucose and insulin levels (Table 1).

We performed unbiased analysis of plasma metabolomics using mass spectroscopy and assessed correlation with insulin sensitivity ($\log S_I$) to identify early signatures of insulin resistance (Supplementary Data Table 2). The metabolite with the highest correlation coefficient was betaine ($r = 0.55$, $P < 0.0005$) (Fig. 1*A*). Betaine concentrations were also significantly lower in IR compared with IS individuals (14% reduction, $P < 0.01$) (Fig. 1*B*). Given the importance of betaine for maintenance of the methionine cycle and systemic metabolism, we hypothesized that reductions in plasma betaine could contribute to metabolic defects associated with insulin resistance. We thus determined the impact of betaine supplementation on in vivo metabolism in mice with diet-induced obesity.

Table 1—Clinical characteristics of human participants

	IS	IR
Subjects, *n* (female/male)	18 (10/8)	23 (13/10)
Age (years)	35.7 ± 2.3	39.4 ± 2.5
BMI (kg/m^2)	25.8 ± 1.0	28.0 ± 1.2
Fasting glucose (mg/dL)	87.9 ± 1.4	92.9 ± 1.8*
Fasting insulin (μU/mL)	4.74 ± 0.42	9.87 ± 1.55**
S_I (IVGTT)	8.12 ± 0.6	2.80 ± 0.3**

Data are summarized as mean ± SEM unless otherwise indicated. Metabolic characteristics of study cohort. IVGTT, intravenous glucose tolerance test. *$P < 0.05$; **$P < 0.0005$.

Betaine Supplementation Improves Glucose Homeostasis

Male C57BL/6 mice were fed chow (10% kcal from fat) (LF) or a matched moderate high-fat diet (45% kcal from fat [HF]) for 16 weeks; high-fat–fed mice were unsupplemented or treated with 1% betaine in water (HF and HF-B, respectively). As expected, high-fat feeding led to greater weight gain, fasting hyperglycemia, and hyperinsulinemia (Table 2). Betaine levels were 50% lower in mice made insulin resistant with high-fat feeding (Table 2), consistent with lower levels in insulin-resistant humans. Lower betaine levels in high-fat–fed mice cannot be attributed to dietary betaine content, as measured betaine content (per gram) is 19% higher in the high-fat diet and calculated daily betaine intake is similar for both groups (Supplementary Fig. 1). Supplementation increased betaine levels by 2.6- and 6.5-fold compared with LF and HF groups (Table 2), respectively, and induced a small decrease in body weight versus HF mice (Fig. 2*A*). Consistent with previous data (15,16), betaine-treated mice had lower fasting insulin (Table 2) and improved glucose tolerance (Fig. 2*B*); plasma insulin was significantly lower in HF-B mice after intraperitoneal glucose (Fig. 2*C*). Insulin tolerance was also improved

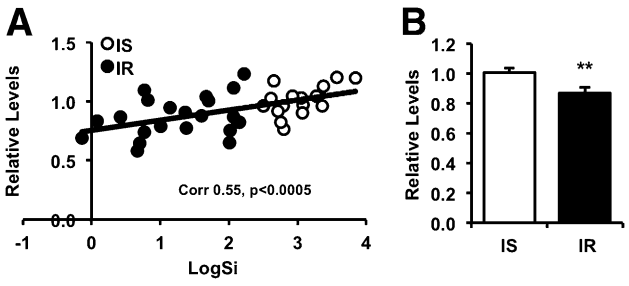

Figure 1—Plasma betaine levels are associated with insulin sensitivity. *A*: Correlation of plasma betaine levels with $\log S_I$ in humans. *B*: Categorical analysis (IR vs. IS) of betaine levels. Data are normalized to median values for IS subjects and presented as mean ± SEM in the categorical analyses. *t* test **$P < 0.01$.

Table 2—Body weight gain during the experiment, cumulative food intake, and fasting plasma glucose, insulin, and betaine levels

	LF	HF	HF-B
Body weight gain (g)	9.9 ± 0.6a	18.3 ± 0.8b	16.6 ± 0.7b
Food intake (kcal/mouse)	ND	1,106 ± 17	1,100 ± 57
Glucose (mg/dL)	74 ± 3a	114 ± 5b	102 ± 4.4b
Insulin (μU/mL)	10.5 ± 2.6a	33.6 ± 2.9b	21.9 ± 1.9c
Betaine (μmol/L)	13.4 ± 1.6a	5.3 ± 0.8a	35.0 ± 5.2b

Data are mean ± SEM from $n = 20$ for HF and HF-B and $n = 15$ for LF from two independent cohorts ($n = 10$ per group for betaine levels). Different letters indicate statistically significant differences between groups, as assessed by one-way ANOVA and post hoc Tukey honestly significant difference test ($P < 0.05$). ND, nondetermined.

(Fig. 2*D*). Pancreatic β-cell area was lower in HF-B versus HF (Fig. 2*E*), indicating that betaine normalized the β-cell expansion with high-fat feeding.

We observed similar results in an independent cohort fed a higher fat diet (60% kcal from fat); betaine supplementation resulted in reduced body weight and leptin, 50% reduction in both fasted and refed insulin, and improved glucose tolerance (Supplementary Fig. 2*A–F*). Moreover, insulin sensitivity was improved, as assessed by insulin tolerance testing, and there was a trend toward a higher glucose infusion rate during hyperinsulinemic-euglycemic clamp (2.1-fold; 5.40 ± 1.36 vs. 2.53 ± 0.76 mg/kg/min/ng/ml, $P = 0.08$) (Supplementary Fig. 2*G* and *H*).

Indirect calorimetry was performed to assess betaine effects on energy homeostasis. Betaine administration increased oxygen consumption by 10% ($P < 0.05$) in both light and dark cycles (Fig. 2*F*). Physical activity did not differ (Supplementary Fig. 3*A*), while respiratory exchange ratio tended to increase in HF-B ($P = 0.056$) (Supplementary Fig. 3*B*). Together with the human data, these observations prompted us to investigate molecular mechanisms mediating betaine action, focusing on HF and HF-B groups.

Betaine Reduces Hepatic Triglyceride Content

Since the liver plays a major role in betaine metabolism, we analyzed betaine effects in this tissue. Supplementation had no major impact in phosphorylated Akt in fasted/refed conditions (Supplementary Fig. 4); however, consistent with previous rodent studies (13–16), betaine decreased liver weight and triglyceride (TAG) content in mice fed either 45% (Fig. 3*A* and *B*) or 60% fat diets (Supplementary Fig. 5*A* and *B*). Fasting plasma TAG levels were also reduced (Fig. 3*C*). Given these robust reductions in hepatic lipid, we used LC-MS to quantify key one-carbon pathway metabolites in liver from 60% fat-fed mice. Hepatic betaine, S-adenosylmethionine, and S-adenosylhomocysteine levels were increased in betaine-supplemented mice (Supplementary Fig. 5*C*); methionine, homocysteine, choline, and cystathionine were not significantly altered.

We next examined potential transcriptional contributions to improved lipid content. Quantitative RT-PCR revealed no major changes in genes regulating lipogenesis or lipid oxidation or in one-carbon metabolic pathway enzymes (Supplementary Fig. 5*D*). However, expression of proinflammatory genes Ccl2, Tnfa, Saa2, and Tlr4 (full gene names in Supplementary Table 1) was consistently downregulated in betaine-treated mice (Fig. 3*D*). Since betaine has antioxidant properties (23,24), we assessed tissue oxidative stress by measuring the ratio of oxidized to reduced glutathione. Betaine supplementation decreased the oxidized-to-reduced glutathione ratio by 20% ($P < 0.05$, Supplementary Fig. 5*E*), suggesting reduced oxidative stress.

Betaine Increases Hepatic and Circulating Fgf21 Levels

Given the robust effect of betaine supplementation on both liver and whole-body metabolism, we hypothesized that a hepatokine was mediating betaine effects. We measured hepatokines known to impact glucose homeostasis and energy expenditure, including Angptl3, Angptl4, Angptl6, Enho, Hgf, Igf1, Sepp1, and Fgf21. Among these, only Fgf21 mRNA was increased (1.6-fold, $P < 0.05$) after betaine treatment (Fig. 3*E*). Circulating Fgf21 levels were also increased in HF-B mice (Fig. 3*F*). For determination of whether increased liver Fgf21 expression was due to a direct transcriptional effect, primary hepatocytes were incubated with betaine (5–25 mmol/L for 5–24 h). Expression of Fgf21 was not increased in any of these conditions (Fig. 3*G* and *H*), despite increases in Fgf21 in response to tunicamycin, a known activator of Fgf21 expression (positive control for these experiments) (Fig. 3*H*). Thus, it is unlikely that effects of betaine on Fgf21 are acute and/or direct. Since Fgf21 is also expressed in extrahepatic tissues, including WAT and brown adipose tissue (BAT), we tested whether betaine modulated Fgf21 expression in these tissues. We found no differences in expression of Fgf21 or its receptor Fgfr1 in either tissue (Supplementary Fig. 6). By contrast, expression of the coreceptor β-klotho was elevated in iWAT (Supplementary Fig. 6), suggesting a potential increase in Fgf21 signaling with betaine supplementation.

Betaine Increases White Adipose Mitochondrial Oxidative Capacity

Circulating Fgf21 exerts most of its whole-body effects via adipose tissue (25–27) and induces a browning program in WAT (28), prompting us to investigate betaine effects on subcutaneous iWAT. Histological analysis showed a shift in adipocyte distribution toward smaller size, with 25% decrease ($P < 0.05$) in mean cell size in HF-B mice (Fig. 4*A*). mRNA expression of Ucp1 and Ppargc1a was increased by 3.9- and 1.5-fold, respectively ($P < 0.05$ for both), with betaine (Fig. 4*B*), but expression of the additional browning markers Cidea or Prdm16 did not differ. Betaine did not alter Ucp1 or Ppargc1a in BAT or epididymal WAT (eWAT) (Fig. 4*C*), indicating that this

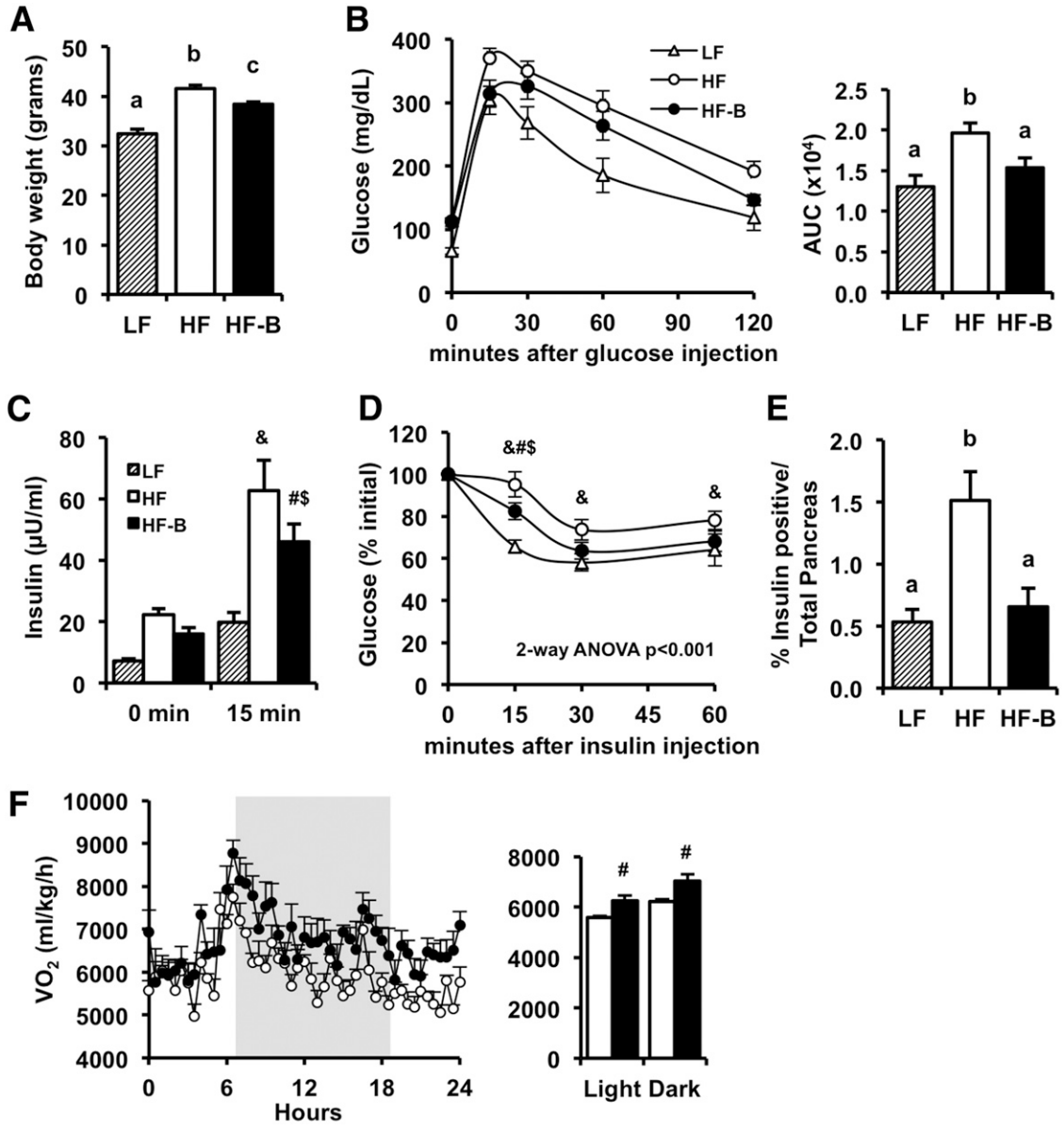

Figure 2—Betaine improves whole-body glucose homeostasis and energy expenditure. Mice were fed the LF, HF, or HF-B diet for 16 weeks. *A*: Body weight at sacrifice. *B*: Glucose tolerance test. Insulin levels before and 15 min after intraperitoneal glucose administration (*C*) and insulin tolerance test (*D*) ($n = 16$ for HF/HF-B; $n = 10$ for LF). *E*: β-Cell area was analyzed by immunohistochemistry ($n = 8$ for HF/HF-B, $n = 3$ for LF). *F*: O_2 consumption was monitored in metabolic chambers at week 10 after starting high-fat feeding; data are summarized as light and dark cycles (gray rectangle) ($n = 7$ for HF/HF-B). Data are mean ± SEM. Different letters indicate statistical significance ($P < 0.05$) between groups after one-way ANOVA and post hoc Tukey honestly significant difference test: $P < 0.05$. Different symbols indicate statistical significance ($P < 0.05$) after two-way ANOVA and post hoc Holm-Sidak test: &HF vs. LF, #HF-B vs. HF, $HF-B vs. LF.

effect was specific for iWAT. Decreased adipocyte cell size and increased oxidative gene expression together suggested that betaine improved oxidative metabolism in iWAT. Consistent with this hypothesis and increased in vivo oxygen consumption, HF-B mice showed enhanced mitochondrial oxidative capacity in iWAT. Specifically, we observed increases in oxygen consumption by 1.5-fold ($P < 0.05$) after the addition of substrates for both *C1* (malate and glutamate) and *CII* (succinate) and in leak-dependent respiration (Fig. 4*D*).

Absence of Fgf21 Abolishes the Beneficial Metabolic Effects of Betaine Supplementation

Betaine-induced improvements in glucose homeostasis, hepatic lipid accumulation, energy expenditure, and iWAT size and oxidative profile are all consistent with a potential role of Fgf21 in mediating betaine effects. To test this hypothesis, we investigated the impact of betaine supplementation in mice null for the Fgf21 gene ($Fgf21^{-/-}$). Male $Fgf21^{-/-}$ mice were fed 45% kcal from fat either without or with 1% betaine in water for 16 weeks

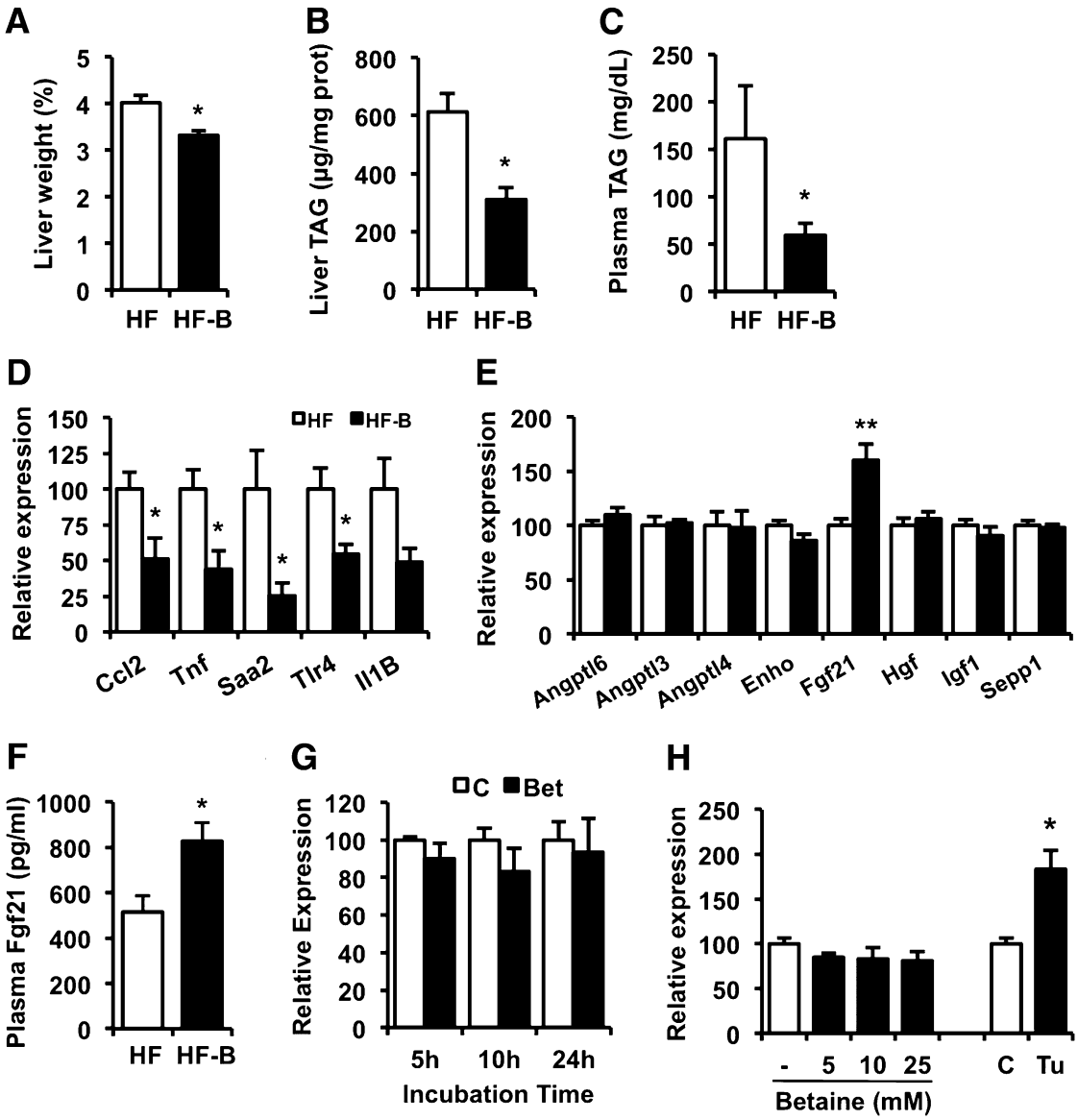

Figure 3—Betaine increases hepatic and circulating Fgf21 levels. *A* and *B*: Liver weight (as % of body weight) and hepatic TAG levels were measured in HF and HF-B mice (n = 20 from two independent cohorts). *C*: Fasting plasma TAG levels were analyzed. *D* and *E*: Hepatic mRNA levels of inflammatory genes and hepatokines were analyzed by RT-PCR (n = 12 from two independent cohorts). *F*: Circulating fasting plasma Fgf21 levels were measured (n = 8). Fgf21 mRNA levels were determined from primary hepatocytes incubated with 10 mmol/L betaine (Bet) for the indicated times (*G*) or with the indicated betaine concentrations for 10 h (*H*); where indicated, hepatocytes were incubated with DMSO (C) or 5 µmol/L tunicamycin (Tu) for 4 h before Fgf21 mRNA levels were analyzed (n = 2 independent experiments performed in triplicate). Data are mean ± SEM. t test $^*P < 0.05$, $^{**}P < 0.01$. Prot, protein.

(KO and KO-B, respectively). Supplementation increased plasma levels by fivefold in KO mice (Fig. 5*A*). Unexpectedly, betaine administration to *Fgf21*$^{-/-}$ mice led to higher body weight (Fig. 5*B*) with no difference in food intake (Fig. 5*C*); these mice also showed increased fasting glucose and numerically higher insulin levels (Fig. 5*D* and *E*). Neither glucose nor insulin tolerance was improved in KO-B vs. KO (Fig. 5*F* and *G*). Furthermore, betaine failed to decrease liver weight, TAG content (Fig. 5*H* and *I*), or inflammatory markers in KO mice (Supplementary Fig. 7). We next analyzed the effects of betaine in iWAT from *Fgf21*$^{-/-}$ mice. As shown in Fig. 5*J*, betaine treatment did not increase expression of Ucp1 or Ppargc1a in iWAT from KO-B versus KO mice. Finally, mitochondrial respiration did not differ between groups (Fig. 5*K*). Together, these data indicate that Fgf21 is necessary for the beneficial effects of betaine to improve glucose homeostasis, reduce liver TAG accumulation, and improve oxidative metabolism in adipose tissue.

DISCUSSION

Betaine is the top-ranking plasma metabolite correlated with insulin sensitivity (S_I) in humans with normal glucose

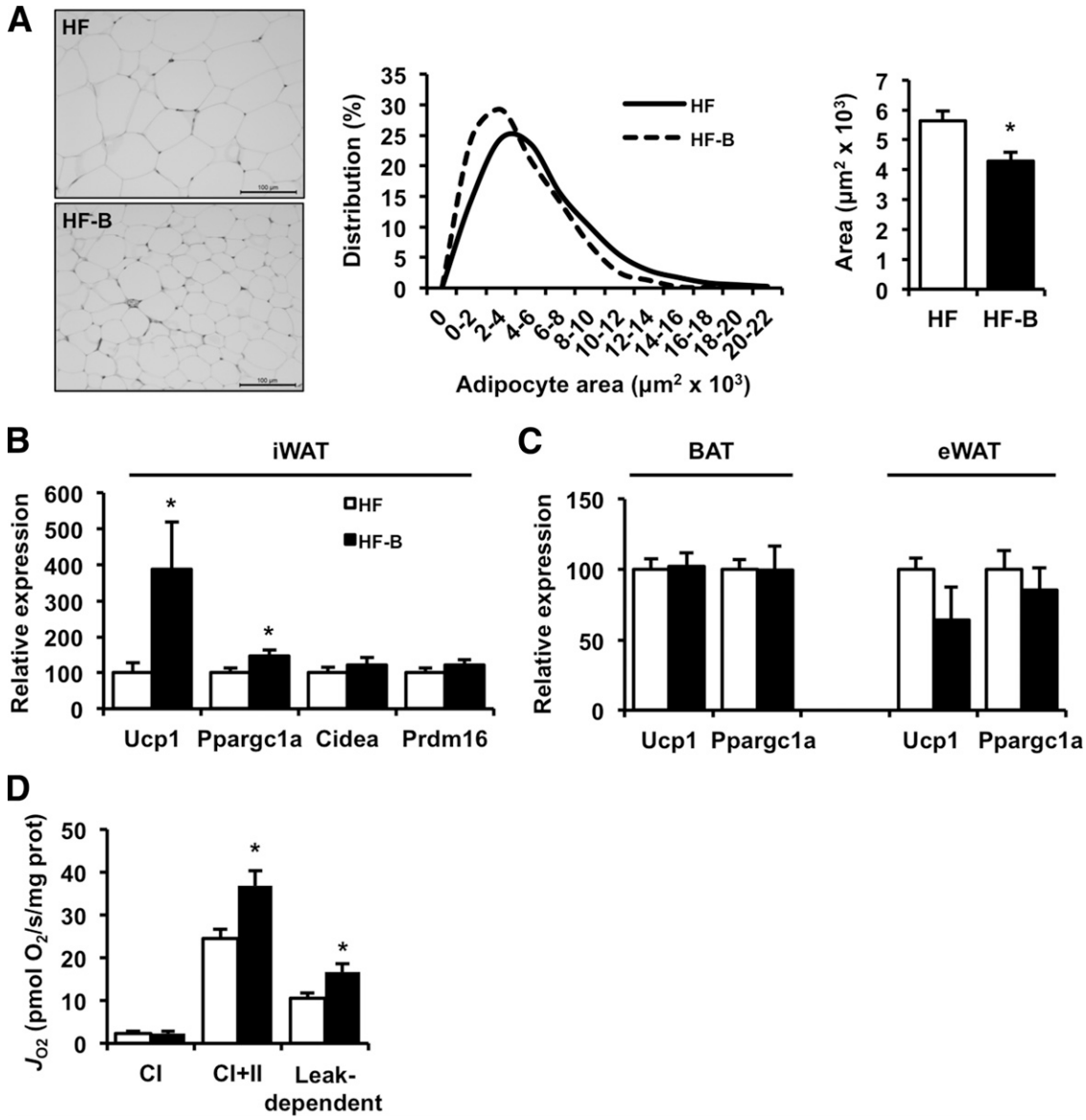

Figure 4—Betaine increases iWAT mitochondrial oxidative capacity. *A*: Adipocyte size was determined in iWAT from HF and HF-B (n = 5) mice; a representative image, cell size distribution, and group means are shown. *B*: iWAT mRNA levels were measured (n = 13–14 from two independent cohorts). *C*: BAT and eWAT mRNA were analyzed (n = 6). *D*: Mitochondrial respiratory capacity was measured in permeabilized iWAT (n = 8). Data are mean ± SEM. t test *$P < 0.05$. Prot, protein.

tolerance, as revealed by unbiased metabolomics analysis. Moreover, increasing betaine levels via long-term dietary supplementation improve systemic metabolism in mice, with prevention of impaired glucose homeostasis and liver fat accumulation, improved insulin sensitivity, increased energy expenditure, and increased oxidative capacity of iWAT, even in mice made obese and insulin resistant with continued high-fat feeding. Thus, plasma betaine concentrations can serve as a biomarker of insulin resistance and a target for prevention and treatment.

More broadly, our data provide support for the emerging concept that one-carbon metabolic pathways may contribute to the pathogenesis of insulin resistance, type 2 diabetes, and related metabolic disease. Prior cross-sectional analysis of data from Framingham Heart Study participants demonstrated inverse correlations between plasma betaine and several phenotypes associated with metabolic disease, including BMI, insulin, blood pressure, and lipids (29). Deficiency of the betaine metabolite dimethylglycine has also been recently associated with higher plasma glucose (30). In new longitudinal prospective studies of Diabetes Prevention Program (DPP) participants, Walford et al. (6) report that higher baseline plasma betaine levels are also associated with a significant reduction in incident diabetes for up to 10 years. Moreover, plasma betaine levels are increased with lifestyle intervention, and increases in betaine predict the success of these interventions to reduce diabetes incidence.

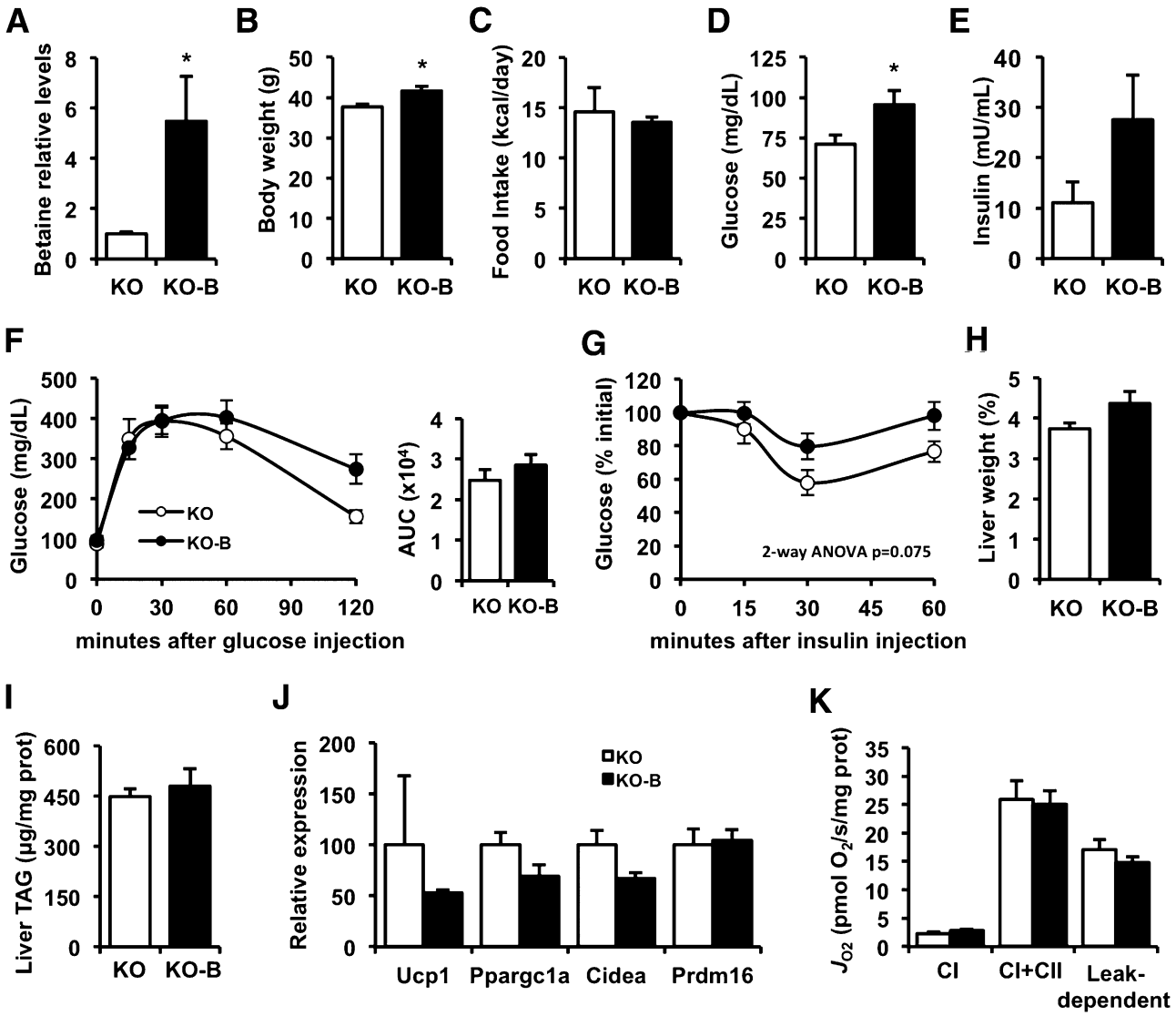

Figure 5—Lack of *Fgf21* abolishes betaine's metabolic effects. *Fgf21*$^{-/-}$ mice were fed a high-fat diet either with no supplement (KO) or with 1% betaine (KO-B). *A–E*: Plasma betaine, body weight, food intake, and fasting plasma glucose and insulin were measured. *F* and *G*: Glucose and insulin tolerance was determined. *H* and *I*: Liver weight (as % of body weight) and liver TAG content were measured. *J*: iWAT mRNA levels of indicated genes were determined. *K*: Mitochondrial respiratory capacity was measured in permeabilized iWAT. Data are mean ± SEM. n = 6–7 mice per group. t test $^*P < 0.05$. AUC, area under the curve. Prot, protein.

While we do not fully understand the mechanisms responsible for these interesting relationships, reduced betaine levels in insulin-resistant humans could reflect reduced intake of betaine-rich foods, as suggested by prior dietary studies (8,31). However, our findings of reduced betaine levels in mice fed a defined high-fat diet, despite similar betaine intake, indicate additional mechanisms. Alterations in intestinal absorption, metabolism of choline-related metabolites by intestinal microbiota (32,33), or osmotic dilution or increased losses could also contribute to reduced plasma betaine in insulin resistance. Additional complexity arises from genetic variation within this pathway, as evidenced by the recently described relationship between variation at the dimethylglycine dehydrogenase (*DMGDH*) locus, insulin resistance, and incident diabetes (30). Additional studies will be required to clarify the relative importance of each of these mechanisms as contributors to reduced betaine levels in human insulin resistance.

Our results support the efficacy of betaine dietary supplementation to improve systemic metabolism. Metabolites from the one-carbon metabolism pathway, including choline, methionine, folic acid, and betaine, singly or in combination, have been used to prevent and treat fatty liver in rodents (15,16,34,35). Our results demonstrate that betaine also yields beneficial effects in nonhepatic tissues, with increases in systemic energy expenditure and enhanced oxidative metabolism in subcutaneous WAT. Thus, both hepatic and extrahepatic mechanisms may contribute to betaine-mediated improvements in insulin sensitivity and glucose tolerance.

One potential mediator of betaine-induced improvements in systemic metabolism is the hormone Fgf21. Fgf21 regulates whole-body glucose and lipid metabolism and is under investigation as a novel metabolic disease therapeutic (36,37). Plasma Fgf21 is elevated during fasting and in response to low protein intake via Peroxisome proliferator-activated receptor alpha (PPARA) and general control nonderepressible 2-dependent regulation (38,39); Fgf21 is also increased in obesity and nonalcoholic fatty liver disease and correlates with hepatic fat, suggesting the possibility of resistance to hormone action (38,40–42). Further increases in Fgf21 in rodents via transgenic overexpression or injection of recombinant Fgf21 improve glucose homeostasis, decrease hepatic lipid, increase whole-body energy expenditure, and reduce body weight (43–45). This is of particular relevance, as our results demonstrate that long-term betaine supplementation yields a more modest increase in both hepatic and circulating Fgf21 by 1.6-fold, while reducing hepatic TAG content. We do not fully understand the mechanisms responsible for betaine-mediated increases in Fgf21 expression or secretion. Our experiments in primary hepatocytes indicate that betaine-induced increases in Fgf21 expression are likely not to be acute or direct but, rather, indirectly mediated by improvements in hepatic insulin action, transcriptional effects on PPARA or related nuclear receptor complexes (38,39,46,47), reduced lipid accumulation, or reduced oxidative stress or via extrahepatic metabolic effects.

Fgf21-induced browning in subcutaneous WAT (28) could contribute to enhanced energy expenditure. Consistent with this effect, betaine treatment increased expression of genes regulating oxidative metabolism, including Ucp1 and Ppargc1a, in subcutaneous WAT; other browning markers were not modified. In parallel, high-resolution respirometry revealed increased iWAT mitochondrial oxidative capacity in betaine-treated mice. While we do not fully understand the mechanisms mediating this effect, increased mitochondrial oxygen consumption and leak-dependent respiration could be mediated in part by the modestly increased Ucp1 and Ppargc1a expression in betaine-treated adipose. Effects of Fgf21 have recently been shown to be Ucp1 independent (48,49), suggesting important contributions of Ppargc1a and other regulators of oxidative metabolism (48,50). Expression of Ucp1 and Ppargc1a did not change with betaine in either BAT or eWAT, consistent with greater susceptibility of iWAT to browning stimuli (51) and Fgf21-mediated induction of thermogenic programs (28). Although we cannot rule out a direct effect of betaine in eWAT (16), the absence of Ucp1 or Ppargc1a induction in iWAT from betaine-treated *Fgf21*$^{-/-}$ mice indicates that Fgf21 is necessary for long-term betaine effects.

Improvements in whole-body glucose homeostasis, liver fat, energy expenditure, and iWAT are all consistent with Fgf21 as a mediator of betaine action. Indeed, betaine supplementation failed to improve metabolic health in *Fgf21*$^{-/-}$ mice, demonstrating that Fgf21 is necessary for betaine metabolic effects. Liver-derived Fgf21 is considered the main source of circulating Fgf21 and responsible for its beneficial systemic effects (40); betaine-induced increases in hepatic Fgf21 mRNA paralleled increased plasma levels. However, we recognize that these mice bear a whole-body knockout, and thus we cannot fully exclude a potential role for extrahepatic Fgf21. Unexpectedly, betaine administration to *Fgf21*$^{-/-}$ mice led to worsening of some metabolic parameters. A plausible explanation is that betaine demethylation ultimately yields glycine, which can be oxidized and provide additional calories. Thus, long-term supplementation could contribute to higher cumulative calorie intake; lack of Fgf21 might impede expenditure of these extra calories, worsening metabolic health. Another possibility is that Fgf21 could regulate betaine metabolism and function; lack of Fgf21 would impede the beneficial effects exerted by long-term betaine supplementation by altering its metabolism.

Additional mechanisms are likely to contribute to betaine-induced improvements in systemic and hepatic metabolism. Betaine is a crucial methyl group donor in the methionine cycle, converting homocysteine into methionine and dimethylglycine in a reaction catalyzed by the enzyme Bhmt. Methionine is sequentially converted into S-adenosylmethionine, and dimethylglycine is further metabolized via the dimethylglycine dehydrogenase to glycine. It is interesting that betaine supplementation increased hepatic content not only of its direct product dimethylglycine, but also of S-adenosylmethionine and its demethylation product S-adenosylhomocysteine (52). Thus, while static assessment of the metabolome does not allow assessment of net cycle flux, these and other downstream metabolites of betaine may be important modulators of betaine effects.

Results from *Bhmt*$^{-/-}$ mice indicate additional complexity (53). These mice show increased liver fat, possibly due to impaired methionine cycle activity and phosphatidylcholine biosynthesis. Surprisingly, *Bhmt*$^{-/-}$ mice are leaner and have improved glucose homeostasis, increased energy expenditure, and higher respiratory exchange ratio, resembling the phenotypic responses to betaine. Interestingly, lack of *Bhmt* also increases betaine accumulation and circulating Fgf21, strongly supporting a role for betaine itself, rather than solely its methyl donor capacity, in increasing Fgf21 levels and improving whole-body metabolism. Further studies will be required to evaluate flux through one-carbon metabolic pathways and to identify the contribution of specific metabolites to betaine-mediated improvements in both hepatic lipid metabolism and Fgf21 secretion.

Another unique feature of betaine is its zwitterion structure; thus, osmolyte functions may also contribute to betaine-mediated improvements in metabolic health. Finally, betaine may also reduce oxidative stress, as demonstrated in multiple tissues and experimental paradigms (23,24), potentially related to betaine-mediated increases

in glutathione biosynthesis and antioxidant responses. In agreement, we found that betaine reduces the ratio of oxidized/reduced glutathione. Reductions in oxidative stress may also be linked to increased complete fatty acid oxidation and AMP kinase activation, as observed with a variety of methyl donors (13,35,54).

The robust effects of betaine supplementation in rodents have prompted human clinical studies focusing on fatty liver and weight loss. Betaine administration to obese humans with nonalcoholic fatty liver disease protected against worsening steatosis and improved hepatic inflammation but did not fully reverse disease (55). In another study, betaine administration to obese individuals on a hypocaloric diet did not enhance weight loss (56). However, these studies were performed on subjects with a preexisting condition and were not designed to examine glucose homeostasis. By contrast, new human data, presented by Walford et al. (6), indicate that betaine levels are not only linked to diabetes risk but also increased in response to lifestyle interventions that successfully reduce diabetes incidence.

Taken together, our human and animal model data support a potential use for long-term betaine supplementation as part of a comprehensive lifestyle intervention aimed at metabolic disease prevention. Future clinical studies will be required to establish whether increased intake of betaine-rich foods and/or long-term betaine supplementation will be a safe and effective strategy to improve metabolic health and prevent type 2 diabetes in at-risk humans.

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Author Contributions. A.E., L.M.-G., M.-E.P., and C.L. designed the experiments, analyzed data, and wrote the manuscript. A.B.G. recruited and characterized metabolic phenotypes in the human cohort. F.R.-A. and F.V. provided *Fgf21*$^{-/-}$ mice and contributed to metabolic phenotyping. V.D.N. performed islet morphometry and together with S.R. assisted with metabolic phenotyping. A.G.-F. and P.M.G.-R. performed high-resolution respirometry. E.L. performed mouse care and contributed to physiologic analysis. J.M.D. performed statistical analysis on metabolomics data. W.G. and R.E.G. performed metabolomics analyses on human and mouse samples, respectively. J.K.K. performed insulin sensitivity analysis. T.B. performed quantitative assay for one-carbon metabolites. All authors assisted in manuscript writing and editing. M.-E.P. and C.L. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Martin BC, Warram JH, Krolewski AS, Bergman RN, Soeldner JS, Kahn CR. Role of glucose and insulin resistance in development of type 2 diabetes mellitus: results of a 25-year follow-up study. Lancet 1992;340:925–929

2. Knowler WC, Barrett-Connor E, Fowler SE, et al.; Diabetes Prevention Program Research Group. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med 2002;346:393–403

3. Gall WE, Beebe K, Lawton KA, et al.; RISC Study Group. alpha-hydroxybutyrate is an early biomarker of insulin resistance and glucose intolerance in a nondiabetic population. PLoS One 2010;5:e10883

4. Newgard CB, An J, Bain JR, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab 2009;9:311–326

5. Wang TJ, Larson MG, Vasan RS, et al. Metabolite profiles and the risk of developing diabetes. Nat Med 2011;17:448–453

6. Walford GA, Ma Y, Clish C, et al. Metabolite profiles of diabetes incidence and intervention response in the Diabetes Prevention Program. Diabetes. 9 February 2016 [Epub ahead of print]. DOI:10.2337/db15-1063

7. Craig SA. Betaine in human nutrition. Am J Clin Nutr 2004;80:539–549

8. Detopoulou P, Panagiotakos DB, Antonopoulou S, Pitsavos C, Stefanadis C. Dietary choline and betaine intakes in relation to concentrations of inflammatory markers in healthy adults: the ATTICA study. Am J Clin Nutr 2008;87:424–430

9. Konstantinova SV, Tell GS, Vollset SE, Nygård O, Bleie Ø, Ueland PM. Divergent associations of plasma choline and betaine with components of metabolic syndrome in middle age and elderly men and women. J Nutr 2008;138:914–920

10. Mato JM, Martínez-Chantar ML, Lu SC. Methionine metabolism and liver disease. Annu Rev Nutr 2008;28:273–293

11. Schwab U, Törrönen A, Meririnne E, et al. Orally administered betaine has an acute and dose-dependent effect on serum betaine and plasma homocysteine concentrations in healthy humans. J Nutr 2006;136:34–38

12. Graham IM, Daly LE, Refsum HM, et al. Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. JAMA 1997;277:1775–1781

13. Song Z, Deaciuc I, Zhou Z, et al. Involvement of AMP-activated protein kinase in beneficial effects of betaine on high-sucrose diet-induced hepatic steatosis. Am J Physiol Gastrointest Liver Physiol 2007;293:G894–G902

14. Kharbanda KK, Mailliard ME, Baldwin CR, Beckenhauer HC, Sorrell MF, Tuma DJ. Betaine attenuates alcoholic steatosis by restoring phosphatidylcholine generation via the phosphatidylethanolamine methyltransferase pathway. J Hepatol 2007;46:314–321

15. Kathirvel E, Morgan K, Nandgiri G, et al. Betaine improves nonalcoholic fatty liver and associated hepatic insulin resistance: a potential mechanism for hepatoprotection by betaine. Am J Physiol Gastrointest Liver Physiol 2010;299:G1068–G1077

16. Wang Z, Yao T, Pini M, Zhou Z, Fantuzzi G, Song Z. Betaine improved adipose tissue function in mice fed a high-fat diet: a mechanism for hepatoprotective effect of betaine in nonalcoholic fatty liver disease. Am J Physiol Gastrointest Liver Physiol 2010;298:G634–G642

17. Jin W, Goldfine AB, Boes T, et al. Increased SRF transcriptional activity in human and mouse skeletal muscle is a signature of insulin resistance. J Clin Invest 2011;121:918–929

18. Planavila A, Redondo I, Hondares E, et al. Fibroblast growth factor 21 protects against cardiac hypertrophy in mice. Nat Commun 2013;4:2019

19. Roberts LD, Souza AL, Gerszten RE, Clish CB. Targeted metabolomics. Current Protocols in Molecular Biology 2012;98:30.2:30.2.1–30.2.24

20. Pfalzer AC, Choi S-W, Tammen SA, et al. S-adenosylmethionine mediates inhibition of inflammatory response and changes in DNA methylation in human macrophages. Physiol Genomics 2014;46:617–623

21. Gnaiger E. Capacity of oxidative phosphorylation in human skeletal muscle: new perspectives of mitochondrial physiology. Int J Biochem Cell Biol 2009;41: 1837–1845
22. Bergman RN, Prager R, Volund A, Olefsky JM. Equivalence of the insulin sensitivity index in man derived by the minimal model method and the euglycemic glucose clamp. J Clin Invest 1987;79:790–800
23. Alirezaei M, Khoshdel Z, Dezfoulian O, Rashidipour M, Taghadosi V. Beneficial antioxidant properties of betaine against oxidative stress mediated by levodopa/benserazide in the brain of rats. J Physiol Sci 2015;65:243–252
24. Jung YS, Kim SJ, Kwon Y, et al. Alleviation of alcoholic liver injury by betaine involves an enhancement of antioxidant defense via regulation of sulfur amino acid metabolism. Food Chem Toxicol 2013;62:292–298
25. Adams AC, Yang C, Coskun T, et al. The breadth of FGF21's metabolic actions are governed by FGFR1 in adipose tissue. Mol Metab 2012;2:31–37
26. Ding X, Boney-Montoya J, Owen BM, et al. βKlotho is required for fibroblast growth factor 21 effects on growth and metabolism. Cell Metab 2012;16:387–393
27. Véniant MM, Hale C, Helmering J, et al. FGF21 promotes metabolic homeostasis via white adipose and leptin in mice. PLoS One 2012;7:e40164
28. Fisher FM, Kleiner S, Douris N, et al. FGF21 regulates PGC-1α and browning of white adipose tissues in adaptive thermogenesis. Genes Dev 2012; 26:271–281
29. Cheng S, Rhee EP, Larson MG, et al. Metabolite profiling identifies pathways associated with metabolic risk in humans. Circulation 2012;125:2222–2231
30. Magnusson M, Wang TJ, Clish C, et al. Dimethylglycine deficiency and the development of diabetes. Diabetes 2015;64:3010–3016
31. Konstantinova SV, Tell GS, Vollset SE, Ulvik A, Drevon CA, Ueland PM. Dietary patterns, food groups, and nutrients as predictors of plasma choline and betaine in middle-aged and elderly men and women. Am J Clin Nutr 2008;88: 1663–1669
32. Tang WHW, Wang Z, Levison BS, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med 2013;368:1575–1584
33. Wang Z, Tang WHW, Buffa JA, et al. Prognostic value of choline and betaine depends on intestinal microbiota-generated metabolite trimethylamine-N-oxide. Eur Heart J 2014;35:904–910
34. Ball CR. Actions of betaine, carnitine and choline on the pattern of hepatic liposis in mice fed a high-fat, low-protein diet. Anat Rec 1964;149:677–689
35. Dahlhoff C, Worsch S, Sailer M, et al. Methyl-donor supplementation in obese mice prevents the progression of NAFLD, activates AMPK and decreases acyl-carnitine levels. Mol Metab 2014;3:565–580
36. Kliewer SA, Mangelsdorf DJ. Fibroblast growth factor 21: from pharmacology to physiology. Am J Clin Nutr 2010;91:254S–257S
37. Gaich G, Chien JY, Fu H, et al. The effects of LY2405319, an FGF21 analog, in obese human subjects with type 2 diabetes. Cell Metab 2013;18:333–340
38. Inagaki T, Dutchak P, Zhao G, et al. Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. Cell Metab 2007;5:415–425
39. Laeger T, Henagan TM, Albarado DC, et al. FGF21 is an endocrine signal of protein restriction. J Clin Invest 2014;124:3913–3922
40. Markan KR, Naber MC, Ameka MK, et al. Circulating FGF21 is liver derived and enhances glucose uptake during refeeding and overfeeding. Diabetes 2014; 63:4057–4063
41. Badman MK, Pissios P, Kennedy AR, Koukos G, Flier JS, Maratos-Flier E. Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. Cell Metab 2007;5:426–437
42. Dushay J, Chui PC, Gopalakrishnan GS, et al. Increased fibroblast growth factor 21 in obesity and nonalcoholic fatty liver disease. Gastroenterology 2010; 139:456–463
43. Xu J, Lloyd DJ, Hale C, et al. Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice. Diabetes 2009;58:250–259
44. Emanuelli B, Vienberg SG, Smyth G, et al. Interplay between FGF21 and insulin action in the liver regulates metabolism. J Clin Invest 2014;124:515–527
45. Coskun T, Bina HA, Schneider MA, et al. Fibroblast growth factor 21 corrects obesity in mice. Endocrinology 2008;149:6018–6027
46. Ong KL, Rye K-A, O'Connell R, et al.; FIELD study investigators. Long-term fenofibrate therapy increases fibroblast growth factor 21 and retinol-binding protein 4 in subjects with type 2 diabetes. J Clin Endocrinol Metab 2012;97:4701–4708
47. Chang MR, He Y, Khan TM, et al. Antiobesity effect of a small molecule repressor of RORγ. Mol Pharmacol 2015;88:48–56
48. Véniant MM, Sivits G, Helmering J, et al. Pharmacologic effects of FGF21 are independent of the "browning" of white adipose tissue. Cell Metab 2015;21:731–738
49. Samms RJ, Smith DP, Cheng CC, et al. Discrete aspects of FGF21 in vivo pharmacology do not require UCP1. Cell Reports 2015;11:991–999
50. Chau MDL, Gao J, Yang Q, Wu Z, Gromada J. Fibroblast growth factor 21 regulates energy metabolism by activating the AMPK-SIRT1-PGC-1α pathway. Proc Natl Acad Sci U S A 2010;107:12553–12558
51. Seale P, Conroe HM, Estall J, et al. Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. J Clin Invest 2011;121: 96–105
52. Deminice R, da Silva RP, Lamarre SG, et al. Betaine supplementation prevents fatty liver induced by a high-fat diet: effects on one-carbon metabolism. Amino Acids 2015;47:839–846
53. Teng YW, Ellis JM, Coleman RA, Zeisel SH. Mouse betaine-homocysteine S-methyltransferase deficiency reduces body fat via increasing energy expenditure and impairing lipid synthesis and enhancing glucose oxidation in white adipose tissue. J Biol Chem 2012;287:16187–16198
54. Xu L, Huang D, Hu Q, Wu J, Wang Y, Feng J. Betaine alleviates hepatic lipid accumulation via enhancing hepatic lipid export and fatty acid oxidation in rats fed with a high-fat diet. Br J Nutr 2015;113:1835–1843
55. Abdelmalek MF, Sanderson SO, Angulo P, et al. Betaine for nonalcoholic fatty liver disease: results of a randomized placebo-controlled trial. Hepatology 2009;50:1818–1826
56. Schwab U, Törrönen A, Toppinen L, et al. Betaine supplementation decreases plasma homocysteine concentrations but does not affect body weight, body composition, or resting energy expenditure in human subjects. Am J Clin Nutr 2002;76:961–967