Choline Supplementation Promotes Hepatic Insulin Resistance in Phosphatidylethanolamine N-Methyltransferase-deficient Mice via Increased Glucagon Action*

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Background: Mice lacking phosphatidylethanolamine N-methyltransferase (Pemt−/−) are glucose- and insulin-sensitive when fed a high fat diet.

Results: Increased plasma glucagon and hepatic gluconeogenesis occurred in Pemt−/− mice fed excess choline.

Conclusion: Supplementation of choline induces insulin intolerance in Pemt−/− mice via increased glucagon action.

Significance: The role of choline should be factored into our thinking about insulin resistance.

Biosynthesis of hepatic choline via phosphatidylethanolamine N-methyltransferase (PEMT) plays an important role in the development of type 2 diabetes and obesity. We investigated the mechanism(s) by which choline modulates insulin sensitivity. PEMT wild-type (Pemt+/+) and knock-out (Pemt−/−) mice received either a high fat diet (HF; 60% kcal of fat) or a high fat, high choline diet (HFHC; 4 g of choline/kg of HF diet) for 1 week. Hepatic insulin signaling and glucose and lipid homeostasis were investigated. Glucose and insulin intolerance occurred in Pemt−/− mice fed the HFHC diet, but not in their Pemt−/− littermates fed the HF diet. Plasma glycerol was elevated in Pemt−/− mice fed the HFHC diet compared with Pemt−/− mice fed the HF diet, concomitant with increased hepatic expression of glucagon receptor, phosphorylated AMP-activated protein kinase (AMPK), and phosphorylated insulin receptor substrate 1 at serine 307 (IRS1-s307). Gluconeogenesis and mitochondrial oxidative stress were markedly enhanced, whereas glucose oxidation and triacylglycerol biosynthesis were diminished in Pemt−/− mice fed the HFHC diet. A glucagon receptor antagonist (2-aminobenzimidazole) attenuated choline-induced hyperglycemia and insulin intolerance and blunted up-regulation of phosphorylated AMPK and IRS1-s307. Choline induces glucose and insulin intolerance in Pemt−/− mice through modulating plasma glucagon and its action in liver.

Phosphatidylcholine (PC)5 is made in mammalian cells via the choline pathway and the phosphatidylethanolamine N-methyltransferase (PEMT) pathway. The PEMT pathway is only quantitatively significant in liver (1). PEMT is responsible for ~30% of hepatic PC biosynthesis, whereas the choline pathway generates the remaining 70% (2, 3). A link since choline and human diseases has attracted attention because choline deficiency was characterized in 1932 (4). Taking advantage of PEMT-deficient (Pemt−/−) mice (5), we have investigated the role of PEMT in mouse metabolism. Despite the lack of PEMT, the levels of hepatic PC and phosphatidylethanolamine in Pemt−/− mice remain unchanged when fed a chow diet (5). Pemt−/− mice develop normally and have normal bile secretion and composition (5). However, secretion of very low density lipoproteins is lower in cultured hepatocytes isolated from Pemt−/− compared with Pemt+/+ mice, demonstrating that PEMT is required for normal lipoprotein secretion (6). Recently we reported that Pemt−/− mice are protected from high fat (HF) diet-induced obesity and insulin resistance (7). This protection was eliminated when excess choline was added to the HF diet, indicating a possible link between choline and obesity/insulin resistance. Choline injection into rats increases plasma glucose, insulin, glucagon, and catecholamine (8–11). However, the mechanism(s) by which choline is linked to insulin resistance should be factored into our thinking about insulin resistance.

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5 The abbreviations used are: PC, phosphatidylcholine; ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; GSK, glycogen synthase kinase; GGT, glucose tolerance test; HF, high fat; HFHC, high fat, high choline; IRS1-s307, insulin receptor substrate 1 at serine 307; ITT, insulin tolerance test; PGC-1α, peroxisome proliferator-activated receptor γ co-activator 1α; PDH, pyruvate dehydrogenase; PDK4, pyruvate dehydrogenase kinase 4; PPAR, peroxisome proliferator-activated receptor; SOD, superoxide dismutase; SREBP, sterol response element-binding protein; TG, triacylglycerol.
sensitivity remains unexplained. We now report that choline causes an increase in plasma glucagon which induces glucose and insulin intolerance in Pemt<sup>−/−</sup> mice.

**EXPERIMENTAL PROCEDURES**

**Animals**—All procedures were approved by the University of Alberta Institutional Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care. Male C57BL/6 Pemt<sup>+/+</sup> and Pemt<sup>−/−</sup> mice had free access to standard chow (LabDiet 5001), HF diet (Bio-Serv F3282), or HF, high choline (HFHC) diet (2.7 g of choline was added per kg of HF diet) for 1 week (7). Five mice were used in each group in the experiments. The basal choline mass in HF diet is 1.3 g/kg. We found that the protective effect of PEMT deficiency on 10-week HF-induced obesity and insulin resistance was abolished when 2.7 g/kg choline was supplemented into the HF diet (7). In the current study we used the same amount of choline supplementation. The glucagon receptor antagonist 2-aminobenzimidazole (50 mg/kg of body weight) was injected intraperitoneally every other day for 1 week into Pemt<sup>−/−</sup> mice fed the HFHC diet. Mice in the control groups received saline. Mice were fasted for 12 h before collection of blood by cardiac puncture. Tissues were stored at −80 °C until further usage.

**Assessment of Lipid, Glycogen, Choline, Glucagon, and Insulin Levels**—Triacylglycerol (TG) mass in liver was measured by gas-liquid chromatography (7, 12). Hepatic glycogen content was measured as described (13). Plasma insulin and glucagon were measured using commercial kits from Meso Scale Discovery. Plasma choline was quantified by Ab Sciei 4000 Qtrap mass spectrometer coupled to an Agilent 1290 Liquid Chromatography system.

**Insulin, Glucose, and Pyruvate Tolerance Tests**—Mice were fasted for 6, 12, or 18 h, followed by intraperitoneal injection of 0.75 unit of human insulin/kg of body weight (Sigma), glucose (2 g/kg of body weight), or 2 g/kg sodium pyruvate (Sigma), respectively. Blood glucose was measured by a glucometer prior and at the indicated times afterward.

**Enzymatic Activity Assays**—Activities of pyruvate dehydrogenase (PDH) and glycerol-3-phosphate acyltransferase (GPTAT) were measured as described (14). Mitochondrial complex I/II activities were assessed by spectrophotometric methods (15).

**Immunoblotting**—Proteins from tissue lysates (cytosol or nuclei) were quantified using the Bradford method (Bio-Rad). Equal amounts of protein were subjected to electrophoresis and immunoblotted with rabbit polyclonal antibodies: PEPCK/PDK4/Mn-superoxide dismutase (Mn-SOD) (1:1000; Abcam); PGC-1α (1:1,000; Santa Cruz Biotechnology); total and phospho-IRS1-s307/AMPK/akt/GSK/AS160/JNK/p38 MAPK (1:1000; Cell Signaling). Anti-tubulin (1:10,000, mouse monoclonal antibody; Sigma) or lamin A (1:1000, goat polyclonal antibody; Santa Cruz Biotechnology) was used as a loading control for cytosol and nuclear proteins, respectively.

**Statistical Analysis**—Data are means of five mice ± S.D. Analysis of variance was performed to compare means unless otherwise specified. A p value of < 0.05 was considered to be significant.

**RESULTS**

Glucose and Insulin Tolerance Are Impaired in Pemt<sup>−/−</sup> Mice Fed the HFHC Diet—Pemt<sup>−/−</sup> mice developed glucose and insulin intolerance when fed the HFHC diet for 10 weeks (7). To explore the link between choline and impaired glucose/insulin tolerance, without development of obesity, Pemt<sup>−/−</sup> and Pemt<sup>+/+</sup> mice were fed the HFHC or HF diet for 1 week. Body weight was not changed significantly in any of the mice (data not shown). However, systemic glucose clearance assessed by a glucose tolerance test (GTT) was significantly delayed in Pemt<sup>−/−</sup> mice fed the HFHC diet compared with littermates on the HF diet (Fig. 1A). No difference in the GTT was found in Pemt<sup>+/+</sup> mice fed either diet (Fig. 1B). An intraperitoneal insulin tolerance test (ITT) showed that Pemt<sup>−/−</sup> mice fed the HFHC diet were less insulin-sensitive compared with their littermates fed the HF diet (Fig. 1C), whereas the diet did not alter the ITT in Pemt<sup>+/+</sup> mice (Fig. 1D).

Choline-induced Hyperglycemia Is Choline-specific—Dimethylethanolamine, structurally related to choline, is converted to phosphatidylcholine, which can substitute for PC in membrane structure (16, 17). Despite this similarity in structure, phosphatidylcholine released the additional choline in the HFHC diet. After 1 week of feeding, dimethylethanolamine did not alter either the GTT (Fig. 1A) or the ITT (Fig. 1C). Thus, choline-induced glucose and insulin intolerance appear to be choline-specific. We next measured the concentration of plasma choline. Plasma choline levels were not significantly changed in Pemt<sup>+/+</sup> mice on the HFHC diet compared with the littermates on the HF diet (Fig. 2A). In contrast, plasma choline levels were significantly elevated in Pemt<sup>−/−</sup> mice on the HFHC diet compared with the littersmates on the HF diet (Fig. 2A). In addition, we also evaluated the effect of acute choline injection on plasma glucose in mice fed a chow diet. A dramatic increase in plasma glucose occurred 10 min after injection of choline both in Pemt<sup>+/+</sup> and in Pemt<sup>−/−</sup> mice (Fig. 2B), and this enhancement was maintained for at least 60 min. Conversely, injection of dimethylethanolamine did not alter plasma glucose (data not shown). Furthermore, to determine whether choline-induced acute hyperglycemia was due to an antagonism of insulin action, choline was injected into mice 2 min prior to insulin. The effect of insulin on lowering plasma glucose was blunted both in Pemt<sup>+/+</sup> and in Pemt<sup>−/−</sup> mice fed the chow diet supplemented with choline (Fig. 2C). To understand whether the choline-induced antagonism of insulin was due to altered coordination between glucagon and insulin, an intraperitoneal glucagon challenge was performed in Pemt<sup>−/−</sup> mice. Plasma glucose levels rapidly increased in Pemt<sup>−/−</sup> mice fed the HFHC diet under both fed (Fig. 2D) and fasted states (Fig. 2E). Glucose clearance was significantly delayed when compared with Pemt<sup>−/−</sup> mice fed the HF diet in both fed (Fig. 2D) and fasted state (Fig. 2E). Moreover, plasma glucagon levels were signifi-
Cyanogenically elevated in *Pemt*−/− mice either after injection of choline or 1 week of the HFHC diet (Table 1). The ratio of glucagon to insulin was significantly higher in *Pemt*−/− mice but not in *Pemt*+/+ mice fed the HFHC diet compared with *Pemt*−/-- littersmates fed the HF diet (Table 1). Plasma insulin levels remained unchanged (Table 1). Thus, there appears to be a specific association between choline-induced hyperglycemia/insulin intolerance and elevated plasma glucagon.
Activation of Hepatic Glucagon Receptor Contributes to Choline-induced Glucose and Insulin Intolerance in Pemt<sup>−/−</sup> Mice Fed the HFHC Diet—Glucagon signals through glucagon receptors, which have a crucial role in mediating hyperglycemia in diabetes (19, 20). Besides kidney, the liver expresses the most abundant glucagon receptors (19). Hence, we investigated a possible role of hepatic glucagon receptors in choline-induced glucose and insulin intolerance in Pemt<sup>−/−</sup> mice. In comparison to Pemt<sup>+/+</sup> mice, hepatic glucagon receptor levels were higher in Pemt<sup>−/−</sup> mice (A). Furthermore, acute choline injection (100 mg/kg of body weight) increased glucagon receptor levels in Pemt<sup>−/−</sup> mice (C). In addition, AMPK phosphorylation was increased in Pemt<sup>−/−</sup> mice (B). TABLE 1: Levels of plasma insulin (I) and glucagon (G) in Pemt<sup>+/+</sup> and Pemt<sup>−/−</sup> mice after 1 week of either high fat or high fat/ high choline feeding

In another set of experiments, choline (100 mg/kg of body weight) was injected intraperitoneally into Pemt<sup>+/+</sup> and Pemt<sup>−/−</sup> mice fed a chow diet, and 10 min later mice were sacrificed followed by the measurement of plasma I and G. The concentration of plasma insulin and glucagon (pg/ml) was measured, and the relative ratio of G/I was then calculated.

| Glucose or insulin | Chronic choline (1 week of feeding) | Acute choline (injection, chow diet) |
|-------------------|-------------------------------------|-------------------------------------|
|                   | Pemt<sup>+/+</sup> | Pemt<sup>−/−</sup> | Pemt<sup>+/+</sup> | Pemt<sup>−/−</sup> |
| I                 | HF        | HFHC     | HF        | HFHC     |
| G                 | 108.0 ± 14.0 | 99.7 ± 22.1 | 135.9 ± 17.9 | 135.9 ± 8.5 |
| G/I               | 6.0 ± 0.5  | 7.1 ± 0.2* | 4.7 ± 0.9  | 7.3 ± 0.4* |
| Increment (fold)  | 1.0 ± 0.1  | 1.2 ± 0.2* | 1.0 ± 0.2  | 1.64 ± 0.3* |

* p < 0.05 compared with high fat diet.

a p < 0.05 compared with mice that only received PBS.

FIGURE 3. HFHC-induced glucose and insulin intolerance in Pemt<sup>−/−</sup> mice involves hepatic glucagon receptors and AMPK. Pemt<sup>−/−</sup> and Pemt<sup>+/+</sup> mice were fed the HF or HFHC diet for 1 week. Representative immunoblots for the hepatic glucagon receptor in Pemt<sup>−/−</sup> mice (A) and Pemt<sup>+/+</sup> mice (E) are shown. Glucagon receptor antagonist, 2-aminobenzimidazole (50 mg/kg of body weight), was injected intraperitoneally on alternate days into Pemt<sup>−/−</sup> and Pemt<sup>+/+</sup> mice, followed by measurement of GTT in Pemt<sup>−/−</sup> (C) and Pemt<sup>+/+</sup> mice (F), as well as ITT in Pemt<sup>−/−</sup> (D) and Pemt<sup>+/+</sup> mice (G). B, representative immunoblots for hepatic phospho-AMPK and total AMPK are shown. Density values of p-AMPK were normalized to t-AMPK levels. a, * p < 0.05, compared with HF-fed mice. b, * p < 0.05, compared with HFHC-fed mice.
FIGURE 4. Impaired hepatic glucose homeostasis in Pemt<sup>−/−</sup> mice fed the HFHC diet. Pemt<sup>+/+</sup> or Pemt<sup>−/−</sup> mice were fed the HF or HFHC diet for 1 week. A and B, hepatic glycogen content in Pemt<sup>−/−</sup> (A) and Pemt<sup>+/+</sup> (B) mice. C and D, a pyruvate tolerance test performed by intraperitoneal pyruvate injection (2 g/kg of body weight) in 18-h fasted Pemt<sup>−/−</sup> (C) and Pemt<sup>+/+</sup> mice (D). E, representative immunoblots for the expression of PEPCK, nuclear PGC-1α in Pemt<sup>−/−</sup> mice. F and G, hepatic PDH activity and representative blots for hepatic protein expression of PDK4 in Pemt<sup>−/−</sup> (F) and in Pemt<sup>+/+</sup> mice (G). Data are mean ± S.D. (error bars) from three independent experiments. *, p < 0.05 compared with littermates on HF diet.

FIGURE 5. Impaired hepatic insulin signaling in Pemt<sup>−/−</sup> mice fed the HFHC diet. Pemt<sup>−/−</sup> mice were fed the HF or HFHC diet for 1 week and then fasted overnight. Insulin was injected intraperitoneally (1 unit/kg of body weight) into mice, and tissues were harvested 5 min later. A, representative immunoblots for the expression of insulin-stimulated phospho-IRS1-s307, phospho-AKT, phospho-GSK-3β, phospho-AS160, relative to the corresponding total (t) proteins. B–E, protein amounts quantified by densitometry of the immunoblots in A. Data are mean ± S.D. (error bars). *, p < 0.05 compared with HF-fed mice.
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FIGURE 6. Excess choline modulates intracellular fatty acid utilization in PEMT−/− mice fed the HF diet. PEMT−/− mice and PEMT+/− mice were fed the HF or HFHC diet for 1 week. A, hepatic TG in 12-h fasted PEMT−/− and PEMT+/−. B, hepatic GPAT activity in both PEMT−/− and PEMT+/− mice. C, incorporation of [14C]oleic acid into hepatic TG in both PEMT−/− and PEMT+/− mice assessed 1 h after intraperitoneal injection of [14C]oleic acid (2 μCi/g). D and E, representative immunoblots for the expression of phospho-p38 MAPK, total MAPK and α-tubulin. D, density values of p-ACC normalized to t-ACC levels. Representative immunoblots for the expression of phospho-p38 MAPK, total MAPK and α-tubulin. E, density values of p-p38 normalized to t-p38 levels. Data are means ± S.D. (error bars). *, p < 0.05, for HFHC diet compared with HF feeding.

The HF diet induces fatty liver in mice fed the HFHC diet compared with littermates fed the HF diet (Fig. 4D). As a result, insulin-stimulated levels of phospho-AKT (Fig. 5, A and C), phospho-GSK (Fig. 5, A and D), and phospho-AS160 (Fig. 5, A and E), the downstream targets of IRS1, were significantly attenuated in PEMT−/− mice fed the HFHC diet compared with the HF diet. Apparently, excess choline impairs hepatic insulin signaling, which contributes to insulin intolerance in PEMT−/− mice.

Fatty Liver Is Ameliorated in PEMT−/− Mice Fed the HFHC Diet—Fatty liver is often associated with insulin resistance (23). The HF diet induces fatty liver in PEMT−/− mice (7). We, therefore, explored whether choline-induced insulin resistance in PEMT−/− mice might be associated with fatty liver. However, hepatic TG content was significantly lower in PEMT−/− mice fed the HFHC diet compared with the HF diet (Fig. 6A), whereas no difference was observed in hepatic TG in PEMT+/− mice (Fig. 6A). Because addition of choline to the HF diet ameliorates fatty liver in PEMT−/− mice fed the HF diet, hepatic TG may not be a causal factor for choline-induced hepatic insulin resistance.

To understand how excess choline reduces hepatic TG levels in PEMT−/− mice, we investigated TG biosynthesis and lipogenesis. The expression of CD36, an important regulator of plasma fatty acid uptake (24), was unaltered (data not shown), suggest-
ing that lower hepatic TG may not be due to altered fatty acid uptake. The activity of hepatic GPAT, a key enzyme for TG biosynthesis, was significantly lower in Pemt−/− mice fed the HFHC compared with the HF diet (Fig. 6B), whereas no difference was observed in Pemt+/+ mice (Fig. 6B). A choline-induced decrease in TG biosynthesis was further demonstrated by in vivo experiments; there was diminished incorporation of [3H]oleic acid into hepatic TG in Pemt−/− mice (Fig. 6C), but not in Pemt+/+ mice, fed the HFHC diet (Fig. 6C). In addition, hepatic acetyl-CoA carboxylase (ACC), a key enzyme for lipogenesis and a downstream target of AMPK, was examined. The phosphorylation/inactivation of ACC was enhanced in Pemt−/− mice fed the HFHC diet (Fig. 6D). Of interest, phospho-p38 MAPK, which has been shown to inhibit hepatic lipogenesis in glucagon-exposed primary hepatocytes (25), was strikingly increased in Pemt−/− mice fed the HFHC diet (Fig. 6E). Thus, choline appears to ameliorate fatty liver in Pemt−/− mice fed the HF diet by reducing TG biosynthesis.

Mitochondrial Oxidative Stress Induced by HFHC Is Improved by an Antagonist of the Glucagon Receptor in Pemt−/− Mice—Aberrant mitochondrial function contributes to insulin resistance (26). However, activity of citrate synthase, an enzyme of the tricarboxylic acid cycle, was unaffected in Pemt−/− mice (Fig. 7A) and in Pemt+/+ mice (Fig. 7B) by either diet. We then examined whether the mitochondrial electron transport chain and/or antioxidative capacity was altered by the HFHC diet. Mitochondrial complex II activity was not altered in Pemt−/− and Pemt+/+ mice by addition of choline to either diet (data not shown). However, the activity of mitochondrial complex I, a major source of reactive oxygen species in mitochondria (27), was slightly lower in Pemt−/− mice (Fig. 7C), but not in Pemt+/+ mice (Fig. 7D) fed the HFHC diet. In contrast, the amount of Mn-SOD was up-regulated in Pemt−/− mice (Fig. 7E), but not in Pemt+/+ mice (Fig. 7F), by the HFHC diet, suggesting a counteractive response to increased oxidative stress in Pemt−/− mice. Furthermore, the reduction in complex I activity in Pemt−/− mice by the HFHC diet was prevented by the glucagon receptor antagonist, 2-aminobenzimidazole (Fig. 7C); 2-aminobenzimidazole also decreased the amount of Mn-SOD (Fig. 7E).

Aberrant mitochondrial fatty acids uptake is a factor associated with mitochondrial oxidative stress (28, 29). It is well known that malonyl-CoA strongly inhibits mitochondrial fatty acid uptake; we, therefore, investigated whether increased mitochondrial oxidative stress occurred in conjunction with decreased malonyl-CoA levels in Pemt−/− mice fed the HFHC diet. Indeed, we observed a dramatic increase in malonyl-CoA (Fig. 7F), suggesting that increased mitochondrial fatty acid oxidation may be due to increased oxidative stress, as evidenced by increased phos-

FIGURE 7. Hepatic mitochondrial oxidative stress and impaired mitochondrial respiratory chain capacity in Pemt−/− mice fed the HFHC diet. Pemt−/− mice and Pemt+/+ mice were fed the HF or HFHC diet for 1 week. One group of Pemt−/− mice on the HFHC diet were injected intraperitoneally with saline or glucagon receptor antagonist, 2-aminobenzimidazole (RA), every other day (50 mg/kg of body weight). A and B, hepatic activity of citrate synthase in Pemt−/− mice (A) and in Pemt+/+ mice (B). C and D, mitochondrial complex I activity in Pemt−/− mice (C) and in Pemt+/+ mice (D). Representative immunoblots show expression of Mn-SOD. E and F, density values of Mn-SOD normalized to tubulin in Pemt−/− mice (E) and in Pemt+/+ mice (F). Protein amounts were quantified by densitometry of the immunoblots. Data are the mean ± S.D. (error bars). a, *, p < 0.05, compared with HF-fed mice. b, **, p < 0.05, compared with HFHC-fed mice.
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FIGURE 8. Glucagon receptor antagonist improves hepatic insulin signaling and restores the level of hepatic malonyl-CoA in *Pemt<sup>−/−</sup>* mice fed the HFHC diet. *Pemt<sup>−/−</sup>* mice were fed the HF or HFHC diet for 1 week. One group of mice fed the HFHC diet were injected intraperitoneally (50 mg/kg of body weight) with 2-aminobenzimidazole (RA) as indicated. A, amount of hepatic malonyl-CoA. B, representative immunobots and densitometry of phospho-c-Jun N-terminal kinase (JNK) normalized to total JNK. C, representative immunobots and densitometry of phospho-IRS1-s307. Data are means ± S.D. (error bars). *a*, p < 0.05, for mice fed HF diet compared with HFHC diet. *b*, p < 0.02, for mice fed HFHC + RA diet compared with mice fed the HFHC diet.

The novel findings are: (i) Addition of choline to the HF diet induced glucose and insulin intolerance in *Pemt<sup>−/−</sup>* mice despite alleviating fatty liver. (ii) The induction was choline-specific, and increased plasma glucagon thereby activating hepatic glucagon receptors/AMPK axis to modulate gluconeogenesis, glucose oxidation and lipid metabolism. (iii) Mitochondrial stress in *Pemt<sup>−/−</sup>* mice caused by excess choline is associated with hepatic insulin resistance; (iv) Antagonism of the glucagon receptor improved the choline-induced hyperglycemia and insulin resistance in *Pemt<sup>−/−</sup>* mice.

**Specificity of Choline on Enhancing Plasma Glucose Levels—**

*Pemt<sup>−/−</sup>* mice depend completely on dietary choline to meet the daily choline requirement (5, 32). In comparison with *Pemt<sup>+/+</sup>* mice fed the HF diet, basal plasma choline levels are significantly reduced in *Pemt<sup>−/−</sup>* mice (Fig. 2A). It could be that the reduced level of basal plasma choline in *Pemt<sup>−/−</sup>* mice reflects greater hepatic uptake of choline for PC biosynthesis (33, 34), and therefore increased hepatic PC content in *Pemt<sup>−/−</sup>* mice, but not in *Pemt<sup>+/+</sup>* mice, after chronic choline supplementation (7). Of interest, HFHC feeding does not change plasma choline levels in *Pemt<sup>+/+</sup>* mice, but significantly enhanced plasma choline levels in *Pemt<sup>−/−</sup>* mice. Plasma glucagon levels are elevated in both types of mice by the HFHC diet, but the increment is about 64% in *Pemt<sup>−/−</sup>* mice, compared with only 20% in *Pemt<sup>+/+</sup>* mice (Table 1). It is currently unknown whether the availability of choline to the pancreas is greater in *Pemt<sup>−/−</sup>* mice than in *Pemt<sup>+/+</sup>* mice, thereby resulting in a different stimulation of glucagon secretion. The difference in the enhanced magnitude of plasma glucagon may contribute to the different response in the expression of hepatic glucagon receptors that is up-regulated only in *Pemt<sup>−/−</sup>* mice, but not in *Pemt<sup>+/+</sup>* mice. In addition, HFHC-induced increase in the ratio of glucagon to insulin seen in *Pemt<sup>−/−</sup>* mice is not observed in *Pemt<sup>+/+</sup>* mice. Apparently, not only plasma glucagon but also the balance between plasma insulin and glucagon associates with choline-induced insulin resistance. It may partially explain why insulin resistance does not develop in *Pemt<sup>+/+</sup>* mice fed the HFHC diet.

To this point, choline injection affects neither plasma glucagon nor the ratio of glucagon to insulin in *Pemt<sup>+/+</sup>* mice. But plasma glucose levels are markedly increased (Fig. 2B), indicative of alternative mechanisms. To our knowledge, the mecha-
FIGURE 9. Proposed mechanism underlying the excess choline-induced hepatic glucose and insulin intolerance in Pemt−/− mice. An increase in choline leads to an increase in plasma glucagon and increase in the expression of hepatic glucagon receptors. The glucagon causes an increase in phospho-AMPK that leads to an increase in PGC-1α. This leads to an increase in PDK4 which inhibits pyruvate dehydrogenase and causes a decrease in glucose oxidation. In parallel, PGC-1α promotes an increase in PEPCK that leads to an increase in gluconeogenesis. As a result of an increase in p-AMPK, the phosphorylation of ACC is increased, leading to a decrease in ACC activity and in the levels of malonyl-CoA. Lower malonyl-CoA promotes an increase in fatty acid uptake in mitochondria. Fewer fatty acids are available for TG biosynthesis, and therefore there is a decrease in fatty liver. The abnormal increase in mitochondrial fatty acids uptake leads to oxidative stress causing an increase in phospho-JNK. As a result, there is an increase in phospho-IRS1. Insulin stimulation causes a decrease in phospho-AKT, phospho-GSK, and phospho-AS160 resulting in insulin resistance in the Pemt−/− mice fed the HFHC diet.

As an endogenous inhibitor of carnitine palmitoyltransferase-1, malonyl-CoA strongly inhibits the rate of mitochondrial fatty acid uptake (37). Supplementation of choline lowered hepatic malonyl-CoA by 55% in Pemt−/− mice compared with littermates fed the HF diet. This could cause enhanced mitochondrial fatty acid uptake and lead to mitochondrial stress (28, 29, 43). This notion is supported by our observations, in which reduction in the activity of mitochondrial complex I, and elevated expression of Mn-SOD expression in conjunction with inactivating AMPK and increasing hepatic malonyl-CoA levels.

Dissociation of Fatty Liver from Choline-induced Hepatic Insulin Resistance in Pemt−/− Mice—Despite the fatty liver in Pemt−/− mice, HF-fed Pemt−/− mice are insulin-sensitive. Excess choline does not improve hepatic TG secretion, yet reduces hepatic TG content and provokes insulin resistance. On the molecular level, supplementation of choline to Pemt−/− mice raises plasma glucagon, which initiates hepatic signaling by activating hepatic AMPK (21, 36), to mobilize fatty acids from TG stores toward mitochondrial uptake (21, 37).

Glucagon suppresses lipogenesis in part via decreased expression of sterol regulatory element-binding protein-1c (SREBP-1c) (25, 38) through the p38 MAPK-dependent inhibitory effect on the SREBP-1c promoter (25). In line with this, we observed increased phosphorylation of p38 MAPK and reduced activities of ACC and GPAT, direct downstream targets of SREBP-1c (39), in livers of Pemt−/− mice fed the HFHC diet. In addition, it has been demonstrated that genetic elimination or antagonism of the glucagon receptor lowers fasting glucose, improves the GTT and pancreatic β-cell function in mice (22, 40), humans (41), and other rodent models (42). Consistently, antagonism of the glucagon receptor in Pemt−/− mice fed the HFHC diet improved insulin sensitivity and enhanced hepatic TG, which occurred in conjunction with inactivating AMPK and increasing hepatic malonyl-CoA levels.

Our data imply because antagonism of the glucagon receptor restored complex I activity and Mn-SOD expression in conjunction with decreased phosphorylation of JNK and IRS1-s307. Of interest, antagonism of the glucagon receptor also prevented the decrease in malonyl-CoA in Pemt−/− mice fed the HFHC diet. Hence, mitochondrial stress that contributes to the choline-induced glucose and insulin intolerance in Pemt−/− mice fed the HF diet.

Proposed Mechanism Underlying the Excess Choline-induced Hepatic Glucose and Insulin Intolerance (Fig. 9)—Our data indicate that choline induces hyperglycemia via increasing plasma glucagon. Consequently, hepatic gluconeogenesis is
enhanced by sequential activation of the glucagon receptor/AMPK/PGC-1α/PEPCK pathway. Concurrently, the increased expression of PGC-1α increases the expression of PDK4, thereby inhibiting PDH activity and impairing glucose oxidation. Furthermore, increased gluconeogenesis decreases the availability of acyl-CoA and glycerol 3-phosphate for TG biosynthesis so that fatty liver is prevented. However, enhanced mitochondrial fatty acid uptake causes oxidative stress so that phospho-c-Jun N-terminal kinase and phosphorylation of IRS1-s-307 are increased. As a result, insulin-mediated signal transduction is blocked (30).

In conclusion, increased provision of choline to Pemt−/− mice fed the HF diet improves fatty liver, but promotes hepatic insulin resistance via increased plasma glucagon. Polymorphisms have been identified in the human Pemt gene (44). Patients with mutations that attenuate Pemt activity are likely to have increased dietary requirements for choline because Pemt is an endogenous source of choline, particularly during pregnancy (45). On the other hand, too much dietary choline supplement may exacerbate glucose and insulin intolerance in humans with obesity or type 2 diabetes. A glucagon receptor antagonist might, therefore, be useful for treatment of type 2 diabetes.

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