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Lack of phosphatidylethanolamine *N*-methyltransferase alters hepatic phospholipid composition and induces endoplasmic reticulum stress

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ABSTRACT

Background & aims: Endoplasmic reticulum (ER) stress is associated with development of steatohepatitis. Phosphatidylethanolamine N-methyltransferase (PEMT) is a hepatic enzyme located on the ER and mitochondria-associated membranes and catalyzes phosphatidylcholine (PC) synthesis *via* methylation of phosphatidylethanolamine (PE). We hypothesized that PEMT deficiency in mice alters ER phospholipid content, thereby inducing ER stress and sensitizing the mice to diet-induced steatohepatitis.
Methods: PC and PE mass were measured in hepatic ER fractions from chow-fed and high fat-fed Pemt$^{-/-}$ and Pemt$^{+/+}$ mice. Proteins implicated in ER stress and the unfolded protein response (UPR) were assessed in mouse livers and in McArdle-RH7777 hepatoma cells that expressed or lacked PEMT. The chemical chaperone 4-phenyl butyric acid was administered to cells and HF-fed Pemt$^{-/-}$ mice to alleviate ER stress.
Results: In chow-fed Pemt$^{-/-}$ mice, the hepatic PC/PE ratio in the ER was lower than in Pemt$^{+/+}$ mice, and levels of ER stress markers, CHOP and BIP, were higher without activation of the UPR. In livers of HF-fed Pemt$^{-/-}$ mice the ER had a lower PC/PE ratio, and exhibited more ER stress and UPR activation. Similarly, the UPR was repressed in McArdle cells expressing PEMT compared with those lacking PEMT, with concomitantly lower levels of CHOP and BIP. 4-Phenyl butyric acid attenuated activation of the UPR and ER stress in McArdle cells lacking PEMT, but not the hepatic ER stress in HF-fed Pemt$^{-/-}$ mice.
Conclusion: PEMT deficiency reduces the PC/PE ratio in the ER and induces ER stress, which sensitizes the mice to HF-induced steatohepatitis.

1. Introduction

The global incidence of non-alcoholic fatty liver disease (NAFLD) is rapidly rising as a complication of obesity and diabetes [1–3]. Recent studies [4,5] have linked NAFLD to a reduced molar ratio of hepatic phosphatidylcholine (PC) to phosphatidylethanolamine (PE). A decrease in the hepatic PC/PE ratio induces steatosis in mice and is also a feature of non-alcoholic steatohepatitis patients [5,6]. Moreover, in mice, the PC/PE ratio strongly predicts liver function and survival after partial hepatectomy [7]. In mice, ~70% of hepatic PC is synthesized *via* the CDP-choline pathway and 30% *via* the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway [8,9]. Inhibition of either pathway results in steatosis [10], which is mainly due to decreased hepatic PC production and impaired very low density lipoprotein secretion [11]. Mice lacking PEMT develop NASH when fed a high-fat (HF) diet for 2 weeks [7,12].

The endoplasmic reticulum (ER) is responsible for the synthesis, folding, trafficking and maturation of proteins, for the synthesis of cholesterol, PC, PE and triacylglycerol and for calcium homeostasis and drug metabolism reviewed in [13,14]. ER stress is associated with chronic syndromes such as obesity, diabetes and fatty liver [15–18]. In response to ER stress, cells activate the unfolded protein response (UPR) to restore the homeostatic equilibrium caused by aberrant protein metabolism [19]. The UPR is mediated by three transducers: protein kinase-like ER kinase, inositol-requiring enzyme-1 (IRE1) and activating transcription factor 6α (ATF6α) as reviewed [19,20], each of which triggers

Abbreviations: ATF6α, activating transcription factor 6α; BIP, glucose-regulated protein 78;; CHOP, C/EBP–homologous protein; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HF, high-fat; IL, interleukin; IRE1, inositol-requiring enzyme-1; McA, McArdle RH-7777 rat hepatoma cells; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBA, 4-phenylbutyric acid; PC, phosphatidylcholine; PDI, protein disulfide isomerase; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase; PERK, protein kinase-like ER kinase; TG, triacylglycerol; TGF-β, transforming growth factor beta; UPR, unfolded protein response; XBP1s, spliced form of X-box binding protein-1.

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distinct and overlapping pathways to re-establish ER homeostasis. PERK inhibits protein translation and decreases protein production *via* phosphorylation of the eukaryotic initiation factor 2α (eIF2α) [21]. PERK activation also increases the pro-apoptotic proteins C/EBP–homologous protein and GADD34 (growth arrest and DNA damage-inducible protein 34) [22,23]. IRE1 activation increases the spliced form of X-box binding protein-1 (XBP1s), which translocates to the nucleus and regulates the production of ER chaperones and proteins involved in ER-associated protein degradation [24]. In addition, the cytosolic domain of IRE1 activates the JNK pathway independently of XBP1s [25]. The third canonical arm of the UPR involves ATF6α which, in response to ER stress, translocates to the Golgi and is cleaved to produce an active form, which migrates to the nucleus and regulates expression of ER chaperones [26].

PEMT is active in the ER and ER-derived mitochondria-associated membranes [27]. Lack of PEMT leads to a decrease in hepatic PC and accumulation of hepatic PE [28]. In livers of *Pemt*$^{-/-}$ mice fed either a choline-deficient diet or a high-fat diet, PC is reduced and PE is increased resulting in a decreased PC/PE ratio [5,7]. We hypothesized that the aberrant ER phospholipid composition in livers of *Pemt*$^{-/-}$ mice leads to ER stress that sensitizes the liver to HF-induced NASH.

2. Materials and methods

2.1. Animal handling, diets and treatments

All procedures were approved by the University of Alberta's Institutional Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care. Male C57BL/6 (backcrossed >7 generations) *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice (8–9 weeks old) [28] were housed with free access to water and were fed either a chow diet (LabDiet, #5001) or a HF diet (Bio-Serv, #F3282). In separate experiments, *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice were administered 4-phenylbutyric acid (PBA, Scandinavian Formulas) or vehicle twice daily (total 1 g/kg body weight/day), by oral gavage during the HF feeding. All mice were fasted for 12 h before sacrifice. Tissues were collected and stored at −80 °C until analysis or preserved in 10% phosphate-buffered formalin for histology.

2.2. Cell culture

McArdle RH-7777 rat hepatoma cells stably expressing the pCI vector (pCI-Empty McA) or human PEMT (PCI-PEMT McA) [29] were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and geneticin G418 (0.2 mg/ml, Invitrogen). Cells were cultured in 10 cm dishes at 37 °C in 5% CO_2, 90% relative humidity. PEMT activity in PCI-PEMT McA cells was similar to that in livers from chow-fed *Pemt*$^{+/+}$ mice [29]**.** In some experiments, cells were incubated for 24 h ± 1 mM PBA.

2.3. Isolation of ER

The protocol for isolation of ER from mouse liver was adapted from Croze and Morre [30]. The purity of ER isolated by this method has been extensively characterized [31]. Briefly, age-matched male *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed a chow or HF diet for two weeks, then fasted overnight and anesthetized by isoflurane. Livers (~1.0 g) were immediately dissected and transferred to 10 ml ice-cold homogenization buffer (0.5 M sucrose, 1% dextran, 37.5 mM Tris, 5 mM $MgCl_2$, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4). The liver was chopped into small pieces and homogenized with a polytron for 30 s at 4000 rpm. The homogenate was centrifuged at 5000 ×*g* for 15 min and the ER fraction was isolated from the supernatant by ultracentrifugation on a sucrose gradient (8 ml 1.3 M sucrose, 8 ml 1.5 M sucrose, 6 ml 2 M sucrose). The ER was collected and washed with ice-cold buffer (55 mM Tris, 5 mM $MgCl_2$, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4). After centrifugation at 90,000 ×*g* for 20 min, the ER pellet was re-suspended in ice-cold buffer (0.25 M sucrose, 10 mM Tris, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) containing a protease inhibitor cocktail (Sigma #P8340). The ER fraction was snap-frozen in liquid nitrogen for further analysis.

2.4. Analytical procedures

The mass of TG was measured by a commercially available kit from Roche Diagnostics according to the manufacturer's protocol. Hepatic PC and PE were isolated by thin-layer chromatography and quantified using a phosphorous assay [32]. Plasma alanine aminotransferase was measured using a commercially available kit (Biotron Diagnostics). Hepatic cytokines and chemokines were quantified at Eve Technologies Corp. (Calgary, Alberta) with the Bio-Plex™ 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), a Milliplex TGF-β 3-plex kit (Millipore, St. Charles, MO, USA) and a Milliplex Mouse Cytokine/Chemokine 32-plex kit (Millipore, St. Charles, MO, USA) according to the manufacturers' protocol. The transforming growth factor-β (TGF-β) 3-plex kit quantified TGF-β1, TGF-β2 and TGF-β3. The 32-plex consisted of Eotaxin, G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte-macrophage colony-stimulating factor), IFNγ, the interleukins (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-15, IL-17, IP-10 (IFN-γ-inducible protein 10), KC (keratinocyte-derived cytokine), LIF (leukemia inhibitory factor), LIX, MCP (monocyte chemotactic protein)-1, MIG (Chemokine (C-X-C motif) ligand 9 (CXCL9), macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, RANTES, TNF α, and VEGF (vascular endothelial growth factor).

2.5. Histology

A portion of the liver was subjected to hematoxylin and eosin (H&E) staining or Tunel staining. Liver slices stained by H&E were scored for steatosis, hepatocellular ballooning, portal inflammation and lobular inflammation using a modified NAFLD scoring system [33]. For Tunel staining, liver slices were stained using APO-BrdU™ TUNEL Assay kit from Life Technologies, following the manufacturer's instructions.

2.6. Real-time quantitative PCR

Total RNA was isolated from snap-frozen livers using TRIzol reagent (Invitrogen). Total RNA was treated with DNase I (Invitrogen) then reverse-transcribed using an oligo(dT)12–18 primer and Superscript II reverse transcriptase (Invitrogen). Primers for CD 68 (forward: GCG GCT CCC TGT GTG TCT GAT; reverse: GGG CCT GTG GCT GGT CGT AG), F4/80 (forward: CCC TCG GGC TGT GAG ATT GTG; reverse: TGG CCA AGG CAA GAC ATA CCA G), collagen I α1 (COL1A1, forward: CTT CAC CTA CAG CAC CCT TGT G; reverse: TGA CTG TCT TGC CCC AAG TTC) and collagen III α1 (COL3A1, forward: TGT CCT TTG CGA TGA CAT AAT CTG; reverse: AAT GGG ATC TCT GGG TTG GG) were purchased from the Institute of Biomolecular Design, University of Alberta. mRNA levels were quantified by real-time quantitative PCR using a Rotor-Gene 3000 instrument (Montreal Biotech) and normalized to cyclophilin (forward: TCC AAA GAC AGC AGA AAA CTT TCG; reverse: TCT TCT TGC TGG TCT TGC CAT TCC) mRNA using a standard curve.

2.7. Immunoblotting

Tissues and cells were homogenized in buffer (25 mM Tris–HCl, 500 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) containing a protease inhibitor cocktail, followed by sonication for 10 s. Proteins were electrophoresed on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were probed with primary