Pioglitazone attenuates hepatic inflammation and fibrosis in phosphatidylethanolamine \(N\)-methyltransferase-deficient mice

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van der Veen JN, Lingrell S, Gao X, Quiroga AD, Takawale A, Armstrong EA, Yager JY, Kassiri Z, Lehner R, Vance DE, Jacobs RL. Pioglitazone attenuates hepatic inflammation and fibrosis in phosphatidylethanolamine \(N\)-methyltransferase-deficient mice. Am J Physiol Gastrointest Liver Physiol 310: G526–G538, 2016. First published January 21, 2016; doi:10.1152/ajpgi.00243.2015.—Phosphatidylethanolamine \(N\)-methyltransferase (PEMT) is an important enzyme in hepatic phosphatidylcholine (PC) biosynthesis. \(Pemt^{-/-}\) mice are protected against high-fat diet (HFD)-induced obesity and insulin resistance; however, these mice develop nonalcoholic fatty liver disease (NAFLD). We hypothesized that peroxisomal proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)) activation by pioglitazone might stimulate adipocyte proliferation, thereby directing lipids from the liver toward white adipose tissue. Pioglitazone might also act directly on PPAR\(\gamma\) in the liver to improve NAFLD. \(Pemt^{-/-}\) and \(Pemt^{+/-}\) mice were fed a HFD with or without pioglitazone (20 mg·kg\(^{-1}\)·day\(^{-1}\)) for 10 wk. \(Pemt^{+/-}\) mice were protected from HFD-induced obesity but developed NAFLD. Treatment with pioglitazone caused an increase in body weight gain in \(Pemt^{-/-}\) mice that was mainly due to increased adiposity. Moreover, pioglitazone improved NAFLD in \(Pemt^{-/-}\) mice, as indicated by a 35% reduction in liver weight and a 57% decrease in plasma alanine transaminase levels. Livers from HFD-fed \(Pemt^{-/-}\) mice were steatotic, inflamed, and fibrotic. Hepatic steatosis was still evident in pioglitazone-treated \(Pemt^{-/-}\) mice; however, treatment with pioglitazone reduced hepatic fibrosis, as evidenced by reduced Sirius red staining and lowered mRNA levels of collagen type I \(\alpha_1\) (Col1a1), tissue inhibitor of metalloproteinases 1 (Timp1), \(\alpha\)-smooth muscle actin (Acta2), and transforming growth factor-\(\beta\) (Tgf-\(\beta\)). Similarly, oxidative stress and inflammation were reduced in livers from \(Pemt^{-/-}\) mice upon treatment with pioglitazone. Together, these data show that activation of PPAR\(\gamma\) in HFD-fed \(Pemt^{-/-}\) mice improved liver function, while these mice were still protected against diet-induced obesity and insulin resistance.

nonalcoholic fatty liver disease; phosphatidylcholine; peroxisomal proliferator-activated receptor-\(\gamma\); steatohepatitis; fibrosis

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Diabetes mellitus. These conditions are strongly connected: obese people frequently develop type 2 diabetes, and insulin-resistant patients are at risk for development of cardiovascular diseases (10, 20). In recent years, we have investigated the role of phosphatidylcholine (PC) synthesis in several aspects of the metabolic syndrome (6, 22, 30, 66).

PC is quantitatively the major phospholipid in mammalian cells and an important component of cell membranes. All nucleated cells can synthesize PC via the CDP-choline pathway (25). The liver is the only organ that has an alternative pathway for synthesizing quantitatively significant amounts of PC by sequential methylation of phosphatidylethanolamine through the action of phosphatidylethanolamine \(N\)-methyltransferase (PEMT) (48). The PEMT pathway is responsible for 30% of the PC synthesized in the liver (8). \(Pemt^{-/-}\) mice are protected against diet-induced obesity and insulin resistance (22). Moreover, when PEMT was deleted in mice lacking the low-density lipoprotein receptor or apolipoprotein E, two models that are susceptible to development of atherosclerosis, atherosclerotic lesion formation was strongly diminished (6, 66). This atheroprotective effect was attributed mainly to reduced secretion of very low-density lipoprotein (VLDL) particles from the liver, resulting in a less atherogenic plasma lipid profile in \(Pemt^{-/-}\) mice. This reduced VLDL secretion, however, was associated with development of fatty liver diseases when \(Pemt^{-/-}\) mice were fed a high-fat diet (HFD) (22). Nonalcoholic fatty liver disease (NAFLD) includes a wide spectrum of liver damage, progressing from steatosis via nonalcoholic steatohepatitis (NASH) to fibrosis and, eventually, cirrhosis or liver failure (1). After 2 wk of HFD feeding, \(Pemt^{-/-}\) mice exhibited steatosis, hepatocellular ballooning, and inflammation. However, no signs of fibrosis were detected (30).

Although PEMT is mainly expressed in the liver, a role for PEMT in white adipose tissue (WAT), with <1% of hepatic expression and activity, has been proposed. PEMT expression is induced during differentiation of 3T3-L1 adipocytes (7), and phosphatidylethanolamine conversion to PC via PEMT is implicated in lipid droplet formation and stability in adipocytes (19). Adipocytes from \(Pemt^{-/-}\) mice seem to be resistant to diet-induced hypertrophy: when mice were fed the HFD, adipocyte diameter was smaller in \(Pemt^{-/-}\) than \(Pemt^{+/-}\) mice (22). We recently reported that PEMT deficiency did not affect differentiation and lipolysis in WAT and only slightly decreased lipogenesis in WAT (14). Thus it appears that the

METABOLIC DISEASES have become a leading cause of deaths in developed countries, where they are often the result of overnutrition. The metabolic syndrome encompasses a cluster of conditions, such as central obesity, hyperglycemia, hyperlipidemia, hypertension, insulin resistance, and hepatic steatosis, which are risk factors for cardiovascular diseases and type 2
resistance to obesity and adipocyte hypertrophy is mostly secondary to the lack of PEMT in the liver.

In this study we investigated a potential role for the peroxisomal proliferator-activated receptor (PPAR)-γ in reducing NAFLD in HFD-fed Pemt−/− mice. PPAR-γ is a member of the PPAR subfamily of nuclear receptors. It is mainly expressed in WAT, where it plays a key role in adipogenesis (55). PPAR-γ is the target of the thiazolidinedione (TZD) class of antidiabetic drugs. TZDs are potent insulin sensitizers that efficiently improve glycemic control in patients with type 2 diabetes mellitus (24, 41). TZDs lower blood glucose levels without increasing pancreatic insulin secretion. It is believed that WAT plays a predominant role in the insulin-sensitizing effect of TZDs. Activation of PPAR-γ enhances the expansability of white adipocytes, thereby shifting intracellular lipid stores from other tissues, such as liver and muscle, in which insulin signaling is impaired, toward WAT (50). Additionally, PPAR-γ activation has anti-inflammatory effects, which contribute to the insulin-sensitizing action of TZDs in WAT (4, 38, 59).

The level of PPAR-γ in normal liver is relatively low (~20% of that in WAT) (12). Nevertheless, PPAR-γ is expressed in hepatocytes, as well as in nonparenchymal cells, including Kupffer cells and hepatic stellate cells (3). In steatotic livers, PPAR-γ expression in hepatocytes is induced (57). Activation of PPAR-γ in hepatocytes stimulates lipogenesis and can result in hepatic lipid accumulation; however, administration of TZDs improves hepatic steatosis in patients with NAFLD (2, 46). This observation suggests that the redistribution of lipids toward WAT after TZD treatment is more pronounced than the increase in hepatic lipid synthesis, resulting in a net reduction of fat accumulation in the liver.

We hypothesized that PEMT deficiency might limit the expansability of WAT and that increasing adipocyte proliferation would allow for increased storage of lipids in WAT, thereby alleviating the burden of excess lipids in livers of Pemt−/− mice. Indeed, we observed that treatment with pioglitazone, a TZD drug, increased adiposity but did not reduce hepatic steatosis. Despite the presence of steatosis in HFD-fed Pemt−/− mice, pioglitazone did improve NAFLD by reducing fibrosis and inflammation. Thus it seems that, in Pemt−/− mice, pioglitazone directly affects the liver, abolishing the detrimental progression from steatosis to NASH and fibrosis.

MATERIALS AND METHODS

Animals. All experimental procedures were approved by the University of Alberta Institutional Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care. Male Pemt+/+ and Pemt−/− mice (backcrossed into C57BL/6 for 7 generations), 8 wk old at the start of the study, were fed a HFD (diet F3282, Bio-Serv) or the HFD supplemented with pioglitazone [0.013% (wt/wt)] ad libitum for 10 wk, and body weight was monitored during this time. Animals were fasted for 12 h before collection of blood by cardiac puncture. Tissues were collected, snap-frozen in liquid nitrogen, and stored at −80°C until further analyses. Samples for histological evaluation were fixed in formalin and subjected to hematoxylin-eosin and Picro-Sirius red (PSR) staining. Liver sections were also immunostained with an antibody against ionized calcium-binding adaptor molecule 1 (Wako Chemicals USA).

In vivo metabolic tests. Mice were fasted for 4 h before insulin tolerance tests and for 12 h before glucose tolerance tests. Subsequently, the mice were injected with bovine insulin (0.5 U/kg body wt ip). Alternatively, glucose (2 g/kg body wt) was administered by oral gavage. Blood glucose levels were measured using a glucose meter (Accu-chek) immediately before and at indicated time points after administration. For in vivo measurement of VLDL production, mice were fasted for 12 h; then they were injected with poloxamer 407 (1 g/kg ip). Blood samples were collected by tail bleeding before and at 1, 2, and 4 h after injection of poloxamer 407. The collected blood samples were used for triacylglycerol (TG) and PC measurements. VLDL-TG production rate was calculated from the slope of TG concentration vs. time curve. Body composition was determined using a whole body composition analyzer (Minispec LF90 II, Bruker, Hamilton, ON, Canada).

Analytical procedures. Plasma nonesterified fatty acids and ketone bodies were quantified using commercially available kits (Wako Chemicals USA). Plasma alanine aminotransferase (ALT) and gamma-glutamyltransferase (ALT) levels were measured using a commercially available kit (Bionrus Diagnostics). Hepatic and plasma TGs were quantified using an enzymatic kit (Roche/Hitachi, Roche Diagnostics). Liver homogenates were centrifuged at 6,000 g for 10 min at 4°C, and the supernatant was centrifuged at 348,000 g for 15 min at 4°C to obtain cytosolic and microsomal fractions. Hydroxylase activity in these fractions was measured using a fluorescence-based assay with 4-methyl umbelliferone as substrate, as described previously (15, 16). Thiobarbituric acid-reactive substances in the liver were measured as described elsewhere (39). For immunoblotting, the liver was homogenized in 25 mM Tris-HCl, 150 mM sodium chloride, 1 EDTA, 1 DTT, and 0.1 PMSF, pH 7.4 containing a protease inhibitor cocktail. Proteins were transferred to a polyvinylidene difluoride membrane, which was probed with primary antibodies against oxidative phosphorylation complexes I–V (catalog no. ab110413, Abcam), carbonic anhydrase (Es) 1 and 3 (29), and glyceraldehyde 3-phosphate dehydrogenase (catalog no. ab8245, Abcam). Immunoreactive proteins were detected using the enhanced chemiluminescence system (GE Healthcare) according to the manufacturer’s instructions, and protein levels were quantified using ImageJ software. RNA isolation, cDNA synthesis, and real-time quantitative PCR were performed as described elsewhere (22). mRNAs were normalized to cyclophilin mRNA. Fibrillar collagen was visualized by PSR staining (5-μm sections, formalin-fixed livers) and confocal microscopy, as described elsewhere (53).

Statistical analysis. Data were analyzed using SAS statistical software (version 9.3, SAS Institute). Values are means ± SE. All data were tested for normal distribution and logarithmically transformed when not normally distributed. Groups were compared using a two-way ANOVA followed by Duncan’s post hoc test. Statistical analyses of the tolerance tests were performed using a two-way ANOVA for repeated measures. Level of significance of differences was P < 0.05.

RESULTS

Pioglitazone increased adiposity in Pemt−/− mice. To evaluate the effects of pioglitazone on whole body metabolism, we monitored body weight during the 10-wk feeding period. After 10 wk of HFD feeding, Pemt+/+ mice gained 11.8 ± 3.9 g body wt, whereas Pemt−/− mice gained only 1.6 ± 0.9 g (Fig. 1A). Treatment with pioglitazone did not affect body weight gain in Pemt+/+ mice. On the other hand, pioglitazone doubled the weight gain in Pemt−/− mice. This was mainly due to increased adiposity, as shown by an increase in the percentage of body fat (from 20.1 ± 1.5% to 24.3 ± 1.5%) upon histological examination (Fig. 1B). Histological examination of visceral WAT showed that, after HFD feeding, adipocytes from Pemt+/+ mice were smaller than those from Pemt−/− mice. After treatment with pioglitazone, the adipocytes from Pemt−/− mice were larger than those from Pemt+/+ mice (Fig. 1C). Plasma nonesterified fatty acid
levels in $\text{Pemt}^{-/-}$ mice were not affected by pioglitazone treatment (Fig. 1D), indicating that the increase in adiposity is not due to reduced lipolysis of TG stored in WAT.

Pioglitazone and PEMT deficiency lowered inflammation and induced browning of WAT. Activation of PPAR$\gamma$ is known to improve insulin sensitivity in part by reducing inflammation in WAT (55). Indeed, treatment of $\text{Pemt}^{+/+}$ mice with pioglitazone seemed to ameliorate adipose tissue inflammation, as indicated by 50% lower mRNA levels of Cd68, F4/80, and tumor necrosis factor-$\alpha$ (Tnfa; Fig. 2, A–C). However, because of large variability in the control group, this did not reach statistical significance. Adipose tissue inflammation was already low in untreated $\text{Pemt}^{-/-}$ mice, and pioglitazone did not further reduce mRNA levels of these inflammatory markers. Another well-known effect of PPAR$\gamma$ activation is browning of WAT, i.e., the induction of genes that are selective for brown adipose tissue (56, 60). An example of such a gene is PPAR$\gamma$ coactivator 1$\alpha$ (Pgc1$\alpha$), a transcriptional coactivator that stimulates mitochondrial biosynthesis. We were surprised to find that the mRNA level of Pgc1$\alpha$ was fourfold higher in WAT from $\text{Pemt}^{-/-}$ than $\text{Pemt}^{+/+}$ mice when fed the HFD (Fig. 2D). Treatment of $\text{Pemt}^{+/+}$ mice with pioglitazone increased expression of Pgc1$\alpha$, but pioglitazone did not further increase Pgc1$\alpha$ levels in $\text{Pemt}^{-/-}$ mice. Similarly, levels of cell death-inducing DNA fragmentation factor $\alpha$-subunit-like effector A (Cidea) and uncoupling protein 1 (Ucp1), both highly expressed in brown adipose tissue and induced by PGC1$\alpha$, were 3- and 20-fold higher in WAT from $\text{Pemt}^{-/-}$ than $\text{Pemt}^{+/+}$ mice, respectively (Fig. 2, E and F). Pioglitazone treatment strongly increased mRNA levels of these genes in both genotypes. As expected, the reduced inflammation and increased browning of WAT correlated with improved insulin sensitivity: $\text{Pemt}^{-/-}$ mice had better insulin sensitivity and glucose tolerance than $\text{Pemt}^{+/+}$ mice when fed the HFD. Pioglitazone improved insulin sensitivity and glucose tolerance in $\text{Pemt}^{+/+}$ mice but had no additional effect in $\text{Pemt}^{-/-}$ mice (Fig. 2, G and H).

Treatment with pioglitazone improved liver function in $\text{Pemt}^{-/-}$ mice. We next evaluated whether these changes in adipose tissue affected the liver in $\text{Pemt}^{-/-}$ mice. We hypothesized that increasing adiposity by activating PPAR$\gamma$ would redirect the flux of fatty acids toward adipose tissue and away from the liver, often referred to as the “lipid-steal hypothesis” (45, 64), thereby alleviating hepatic steatosis in $\text{Pemt}^{-/-}$ mice. Indeed, after 10 wk of HFD feeding, $\text{Pemt}^{-/-}$ mice showed a nearly fivefold increase in hepatic TG and hepatomegaly (Fig. 3, A–D). Liver weight of $\text{Pemt}^{-/-}$ mice was 11.4% of total body weight compared with 3.3% in $\text{Pemt}^{+/+}$ mice. Pioglitazone treatment reduced liver weight in $\text{Pemt}^{-/-}$ mice to 7.4% of total body weight (Fig. 3A). However, pioglitazone did not reduce the concentration of TG in livers of $\text{Pemt}^{-/-}$ mice (Fig. 3C). Nevertheless, because of the reduction in liver size in pioglutazone-treated $\text{Pemt}^{-/-}$ mice, total amounts of TG were 40% lower in livers of these pioglitazone-treated than untreated $\text{Pemt}^{-/-}$ mice (Fig. 3C). Histological examination suggested that treatment with pioglitazone reduced the size of lipid droplets in livers of $\text{Pemt}^{-/-}$ mice (Fig. 3D). Overall liver function of $\text{Pemt}^{-/-}$ mice was improved after pioglitazone treatment, as indicated by 60% lower plasma ALT levels in pioglitazone-treated than untreated $\text{Pemt}^{-/-}$ mice (Fig. 3E).
However, liver function was not completely normalized, as plasma ALT levels were still higher in treated Pemt-/- mice, in which ALT was undetectable. TG turnover was not affected by pioglitazone treatment. To determine the mechanism by which pioglitazone improved liver function and reduced the total amount of TG in the liver, we investigated turnover and secretion of hepatic TG. One of the major reasons for TG accumulation in livers of Pemt-/- mice is reduced capacity for secretion of VLDL particles (37). Therefore, we measured plasma TG and the secretion rate of VLDL-TG. Although plasma TG was not significantly reduced in Pemt-/- mice, the rate of VLDL-TG production was 70\% lower in Pemt-/- than Pemt-/+ mice (Fig. 4, A and B). Pioglitazone did not restore the capability for secretion of TG in Pemt-/- mice. Moreover, we measured protein levels of Ces-1/esterase (Es)-x and Ces-3/TG hydrolase, which are present in the lumen of the endoplasmic reticulum and play important, but opposite, roles in VLDL-TG secretion (9, 44, 45).

Fig. 2. Markers of inflammation and browning of white adipose tissue (WAT). A–F: relative mRNA levels of genes involved in inflammation and browning of WAT from Pemt-/+ and Pemt-/- mice fed the HFD or pioglitazone-supplemented HFD. Tnfa, tumor necrosis factor-α; Cidea, cell death-inducing DNA fragmentation factor α-subunit-like effector A; Ucp1, uncoupling protein 1. G and H: insulin and glucose tolerance tests. Values are means ± SE (n = 6 per group). a,b Bars or symbols that do not share a letter are significantly different (P < 0.05).
reducing lipid accumulation in livers of if pioglitazone increased oxidation of fatty acids, thereby liver function. excluded as a mechanism by which pioglitazone improved produced plasma levels of ketone bodies in Pemt mice after HFD feeding. A–C, liver weight and hepatic triacylglycerol (TG) in Pemt+/+ and Pemt−/− mice fed the HFD or pioglitazone-supplemented HFD. D: representative images of hematoxylin-eosin-stained livers. E: plasma alanine aminotransferase (ALT) levels of Pemt+/+ and Pemt−/− mice fed the HFD or pioglitazone-supplemented HFD. Values are means ± SE (n = 6 per group). a,b,c Bars that do not share a letter are significantly different (P < 0.05).

Fig. 3. Pioglitazone improved liver function in Pemt−/− mice after HFD feeding. A–C, liver weight and hepatic triacylglycerol (TG) in Pemt+/+ and Pemt−/− mice fed the HFD or pioglitazone-supplemented HFD. D: representative images of hematoxylin-eosin-stained livers. E: plasma alanine aminotransferase (ALT) levels of Pemt+/+ and Pemt−/− mice fed the HFD or pioglitazone-supplemented HFD. Values are means ± SE (n = 6 per group). a,b,c Bars that do not share a letter are significantly different (P < 0.05).

58). Especially the level of Ces-3 was lower in livers of Pemt−/− than Pemt+/+ mice and was increased by pioglitazone treatment; however, there was no difference in microsomal hydrolase activity among the different groups of animals (Fig. 4, C and D). Therefore, increased VLDL-TG secretion can be excluded as a mechanism by which pioglitazone improved liver function.

Similarly, we investigated fatty acid oxidation to determine if pioglitazone increased oxidation of fatty acids, thereby reducing lipid accumulation in livers of Pemt−/− mice and improving liver function. Plasma ketone bodies were not different between Pemt+/+ and Pemt−/− mice. Pioglitazone reduced plasma levels of ketone bodies in Pemt+/+ mice but had no effect in Pemt−/− mice (Fig. 5A). In addition, pioglitazone reduced the mRNA levels of acyl-coenzyme A oxidase (Acox) in Pemt+/+ mice but did not lower the mRNA levels of carnitine palmitoyltransferase 1α (Cpt1α) (Fig. 5, B and C). Pioglitazone had no effect on mRNA levels of Acox or Cpt1α in Pemt−/− mice. Protein levels of the mitochondrial oxidative phosphorylation complexes I–V were reduced by PEMT deficiency, suggesting that oxidative capacity of these livers was reduced. Pioglitazone did not change amounts of these proteins in Pemt+/+ or Pemt−/− mice, except for a slight reduction of complex I in Pemt+/+ mice (Fig. 5, D and E). In addition, we measured TG hydrolase activity in cytosolic fractions of the livers. Adipose triglyceride lipase (ATGL), a cytosolic lipase, is important for fatty acid oxidation and activation of PPARs (42). However, levels of mRNA encoding ATGL were not different among the four groups, whereas mRNA levels of the Gi/Gi switch 2 gene (G0s2), an inhibitor of ATGL activity (61), were strongly reduced in Pemt−/− mice (Fig. 5, G and H). Cytosolic TG hydrolase activity was slightly higher in livers of Pemt−/− than Pemt+/+ mice, and pioglitazone reduced this hydrolase activity only in Pemt−/− mice (Fig. 5F). It is important to note that this measurement is not specific for ATGL, since other lipases are also present in the cytosol. Additionally,
the larger amount of TG in homogenates of \textit{Pemt}^{-/-} mice might compete with 4-methyl umbelliferyl heptanoate, leading to underestimation of hydrolase activity in these samples. This, however, would affect the comparison between \textit{Pemt}^{+/+} and \textit{Pemt}^{-/-} mice, not the comparison between treated and untreated animals. Overall, these measurements indicate that pioglitazone did not increase fatty acid oxidation in livers of \textit{Pemt}^{-/-} mice.

We also measured expression of genes involved in de novo lipogenesis. Although mRNA levels of sterol regulatory element-binding protein 1c; \textit{Acc}, acetyl-CoA carboxylase; \textit{Dgat2}, diacylglycerol O-acethyltransferase 2. Values are means \pm SE (\(n = 6\) per group). \(a,b\)-Bars that do not share a letter are significantly different (\(P < 0.05\)).
Fig. 5. Pioglitazone did not increase the capacity for fatty acid oxidation in livers of Pemt<sup>−/−</sup> mice. A: plasma ketone bodies of Pemt<sup>+/+</sup> and Pemt<sup>−/−</sup> mice fed the HFD or pioglitazone-supplemented HFD. B and C: hepatic mRNA levels of genes involved in fatty acid oxidation [carnitine palmitoyltransferase 1α (Cpt1α) and acyl-coenzyme A oxidase (Acox)]. D: immunoblot of mitochondrial oxidative phosphorylation complexes I–V in livers from Pemt<sup>+/+</sup> and Pemt<sup>−/−</sup> mice fed the HFD or pioglitazone-supplemented HFD. E: quantification of immunoblot in D. F: hydrolase activity in cytosolic fractions of livers from Pemt<sup>+/+</sup> and Pemt<sup>−/−</sup> mice fed the HFD or pioglitazone-supplemented HFD. G and H: hepatic mRNA levels of genes involved in TG hydrolysis [adipose triglyceride lipase 1 (Atgl) and G0G1 switch 2 (G0S2)]. Values are means ± SE (n = 6 per group). aa,ab Bars that do not share a letter are significantly different (P < 0.05).
ment-binding protein 1c (Srebp1c), the master regulator of lipogenesis, was lower in Pemt-/- mice and even further reduced by pioglitazone in Pemt-/- mice, its target genes Fas and acetyl-CoA carboxylase 1 (Accl) were not different between the groups, suggesting that there is no difference in de novo lipogenesis (Fig. 4, E–G). Similarly, mRNA levels of diacylglycerol O-acyltransferase 2 (Dgat2) were not different between treated and untreated mice, indicating that TG formation was not affected by pioglitazone (Fig. 4H).

Together, these results indicate that pioglitazone did not increase TG secretion or oxidation, nor did pioglitazone decrease de novo lipogenesis. Consequently, these pathways can be excluded as a mechanism by which pioglitazone improved liver function.

Hepatic inflammation and fibrosis were reduced in Pemt-/- mice after treatment with pioglitazone. PEMT deficiency not only causes accumulation of TG in the liver, but the livers become inflamed even after 1 wk of HFD (30). We investigated whether treatment with pioglitazone diminished the development of NASH in Pemt-/- mice. We initially measured mRNA levels of TNFα, an inflammatory cytokine, and CD68 and CD11c, a general and a proinflammatory macrophage marker, respectively. The levels of all three mRNAs were markedly higher in Pemt-/- than Pemt+/- mice and were reduced by 55% after treatment with pioglitazone (Fig. 6A). Similarly, immunohistochemical staining for ionized calcium-binding adaptor molecule 1 showed macrophage infiltration in livers of Pemt-/- mice, which was reduced after pioglitazone treatment (Fig. 7A). mRNA levels of monocyte chemotactic protein 1 (Mcp1) were also increased in Pemt-/- compared with Pemt+/- mice, but pioglitazone did not reduce the expression of this monocyte-recruiting cytokine (Fig. 6A). NAFLD encompasses a wide spectrum of severity of the disease, ranging from simple steatosis, via steatohepatitis, to the more severe fibrosis and, eventually, cirrhosis. Therefore, we evaluated if the reduction in inflammation by pioglitazone would inhibit the development of hepatic fibrosis. Indeed, HFD feeding of Pemt-/- mice resulted in hepatic fibrosis as assessed by PSR staining, and the amount of fibrillar collagen was lower when Pemt-/- mice were treated with pioglitazone (Fig. 7B). Consistent with this staining, mRNA levels of collagen type Iα1 (Coll1a1), tissue inhibitor of metalloproteinase 1 (Timp1), α-smooth muscle actin (Acta2), and transforming growth factor-β (Tgf-β) were strongly induced in livers of Pemt-/- mice, and treatment with pioglitazone reduced these levels by 45–70% (Fig. 6B). Moreover, oxidative stress was also reduced after pioglitazone treatment, as indicated by lower amounts of hepatic thiobarbituric acid-reactive substances (Fig. 6C), a measure of lipid peroxidation, and lower mRNA levels of Ucp2, NADH oxidase 2 (Nox2), and heme oxygenase 1 (Hmox1) (Fig. 6D). Protein levels of CCAAT/enhancer-binding protein homologous protein (CHOP) were also lower in pioglitazone-treated Pemt-/- mice, indicating reduced endoplasmic reticulum stress (Fig. 6E). Together, these data show that pioglitazone clearly improved several aspects of liver disease in Pemt-/- mice.

**DISCUSSION**

We were able to demonstrate improved liver function by treating Pemt-/- mice with pioglitazone to activate PPARγ. Although pioglitazone redirected the flux of lipids toward WAT, which alleviated the lipid burden on the liver to a small extent, hepatic steatosis was not prevented in Pemt-/- mice. Nevertheless, treatment of Pemt-/- mice with pioglitazone improved hepatic function, decreased the size of the liver, and reduced the amount of hepatic fibrosis and inflammation. PPARγ activation in WAT. PPARγ expression is essential for adipocyte differentiation (54). PPARγ is a master regulator of adipocyte lipid metabolism (49), and activation of PPARγ is associated with fat accretion in WAT due to enhanced uptake and storage of circulating lipids (26, 27). Most of the insulin-sensitizing effects of TZDs are due to PPARγ activation in WAT: when PPARγ is overexpressed, specifically in adipocytes, whole body insulin sensitivity is improved to an extent similar to that after TZD treatment (51). On the other hand, insulin sensitivity was not improved upon TZD treatment of adipose-specific Pparγ-/- mice (18). The mechanism by which adipocyte PPARγ activation improves whole body insulin sensitivity is that PPARγ enhances the expandability of white adipocytes, increasing fat storage in a metabolically safe compartment. This results in a reduction in circulating lipids and less exposure of nonadipose tissues to lipotoxicity, thereby increasing insulin sensitivity (28, 50). Therefore, we hypothesized that activation of PPARγ by pioglitazone would improve NAFLD in Pemt-/- mice by redirecting lipids away from the liver toward WAT. Indeed, pioglitazone increased adiposity in these mice, but this was not sufficient to prevent TG accumulation in the liver. Nevertheless, the partial redirection of fatty acids away from the liver seemed to be sufficient to prevent the hepatomegaly that occurs in HFD-fed Pemt-/- mice.

Besides increasing the lipid storage capacity in WAT, PPARγ activation also reduces WAT inflammation, which improves insulin sensitivity. Activation of PPARγ reduces production of cytokines, such as TNFα, interleukin-6, and monocyte chemoattractant protein-1, and reduces accumulation of inflammatory macrophages (55). Hepatic lipid accumulation. NAFLD comprises a wide spectrum of liver diseases, ranging from simple steatosis to liver fibrosis and cirrhosis. NAFLD has become an important health issue because of its high prevalence. The role of PPARγ in NAFLD is controversial. PPARγ is a lipogenic factor, and activation of PPARγ induces TG accumulation in hepatocytes: rosiglitazone, a TZD, induced lipid accumulation in wild-type hepatocytes; however, this effect was completely abrogated in PPARγ-deficient hepatocytes (33). Moreover, hepatocyte-specific Pparγ-/- mice are protected from HFD-induced hepatic steatosis (33). These studies indicate that activation of PPARγ would be detrimental for the development of NAFLD. The controversy is that administration of TZDs to humans is actually beneficial in the treatment of NAFLD (2, 46). This latter finding supports the idea that TZDs mainly act on PPARγ in WAT, with secondary effects on hepatic lipid metabolism. This conclusion is further underscored by the observations that TZDs reduce blood glucose and liver TG levels in muscle-specific and liver-specific Pparγ-/- mice, showing that PPARγ activation in these tissues makes only a minor contribution to the beneficial effects of TZD treatment. Thus, although PPARγ is a lipogenic factor, the effects of PPARγ activation in WAT overpower the lipogenic effects in liver, resulting in increased TG storage in adipose tissue, rather than in the liver. Indeed, we did observe an increase in adiposity of
Fig. 6. Reduced markers of hepatic inflammation, fibrosis, oxidative stress, and endoplasmic reticulum stress in livers of Pemt<sup>−/−</sup> mice. A and B: hepatic mRNA levels of genes involved in inflammation, fibrosis, and oxidative stress. Mcp1, monocyte chemotactic protein 1; Col1a1, collagen type I; Timp1, tissue inhibitor of metalloproteinase 1; Tgf-β, transforming growth factor-β; Acta2, α-smooth muscle actin. C: hepatic concentration of thiobarbituric acid-reactive substances (TBARS) as a marker for lipid peroxidation. D: mRNA levels of NADH oxidase 2 (Nox2), Ucp2, and heme oxygenase 1 (Hmox1). E: immunoblot of the endoplasmic reticulum stress marker CCAAT/-enhancer-binding protein homologous protein (CHOP) in livers from Pemt<sup>+/+</sup> and Pemt<sup>−/−</sup> mice fed the HFD or pioglitazone-supplemented HFD. Bands were quantified using ImageJ software. Values are means ± SE (n = 6 per group). a,b,c Bars that do not share a letter are significantly different (P < 0.05).
Pemt−/− mice upon PPARγ activation, but this was not sufficient to prevent hepatic steatosis. However, because of the smaller size of the liver, the total amount of TG in the liver was lower in pioglitazone-treated than untreated Pemt−/− mice. We did not see a decrease in lipogenic gene expression (mRNA for Srebp1c, Fas, Acc, and Dgat2) upon pioglitazone treatment, suggesting that hepatic de novo lipogenesis and TG formation were not changed by the treatment. We previously showed that hepatic lipid accumulation in the absence of PEMT is mainly caused by impaired VLDL secretion (22, 36, 37). This is likely due to insufficient hepatic PC synthesis, since the same phenomenon is observed in mice lacking CTP:phosphocholine cytidylyltransferase-α (21, 35), the rate-limiting enzyme of the choline pathway for PC synthesis, as well as in rodent models that were fed a methionine- and choline-deficient diet (62). Treatment with pioglitazone did not restore normal VLDL secretion in Pemt−/− mice; therefore, it was not surprising that pioglitazone treatment did not prevent hepatic steatosis. Thus we conclude that 1) the reduction in the total amount of TG in livers of Pemt−/− mice upon pioglitazone treatment was solely due to fat redistribution toward WAT and 2) the improved liver function was due to other beneficial effects of PPARγ activation independent of hepatic TG concentrations.

Hepatic inflammation and fibrosis. In our study, hepatic steatosis was not prevented by pioglitazone treatment in Pemt−/− mice, but we did observe improvements in hepatic...
inflammation and markers of fibrosis, suggesting that liver cells other than hepatocytes are directly affected by pioglitazone and are involved in the beneficial effects of pioglitazone treatment in these mice. Although the increase in PPARγ expression in rodent models of fatty liver and in NAFLD patients occurs mainly in hepatocytes, PPARγ is also expressed in nonparenchymal cells in the liver, including Kupffer cells and hepatic stellate cells (3). Activation of Kupffer cells and secretion of cytokines play important roles in the development of hepatic inflammation and fibrosis, since inflammatory cytokines can activate hepatic stellate cells, which is the critical event in hepatic fibrosis. Activated hepatic stellate cells are the source of excessive production of extracellular matrix, leading to hepatic fibrosis (13, 40).

PPARγ is expressed in macrophages, including Kupffer cells, and activation of PPARγ has anti-inflammatory effects (5, 11). PPARγ ligands control the expression of genes implicated in the inflammatory response by negatively influencing inflammatory cytokine production and cell recruitment to inflammatory sites (23, 47, 52). In agreement with these findings, in livers of Pemt−/− mice, we observed a massive increase in levels of mRNAs encoding TNFα, CD68, and CD11c that was reduced by ≥40% after treatment with pioglitazone. Moreover, immunohistochemical staining showed a striking reduction in the degree of macrophage infiltration in treated vs. untreated livers from Pemt−/− mice. This anti-inflammatory effect of pioglitazone most likely inhibited the activation of hepatic stellate cells, resulting in the improvement in fibrosis that we observed in the Pemt−/− mice. Additionally, there is almost certainly an effect of PPARγ activation directly on hepatic stellate cells. PPARγ is expressed in hepatic stellate cells, and its expression and transcriptional activity are strikingly reduced during the process of activation from the quiescent phenotype to a highly proliferative and fibrotic phenotype (17, 31, 32, 63). Moreover, ectopic expression of PPARγ or activation of PPARγ with TZDs prevents activation of hepatic stellate cells and can even restore activated hepatic stellate cells to the quiescent state (31, 43, 65). Accordingly, we observed 60% and 45% reduction in hepatic Col1a1 and Timp1 mRNA levels, respectively, suggesting decreased accumulation of activated hepatic stellate cells and collagen production, as well as increased matrix degradation, thereby preventing or reversing hepatic fibrosis development. This was indeed the case, as PPARγ overexpression improved inflammation and fibrosis in the liver (34), we demonstrate here that treatment of HFD-fed Pemt−/− mice with pioglitazone exerts anti-inflammatory and antifibrotic effects in the liver.

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No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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