



# Hepatic Phosphatidylethanolamine *N*-Methyltransferase Expression Is Increased in Diabetic Rats<sup>1</sup>

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## Abstract

Phosphatidylcholine is an essential phospholipid that is synthesized by 2 different pathways, the CDP-choline pathway and the methylation of phosphatidylethanolamine by phosphatidylethanolamine *N*-methyltransferase (PEMT). Recent studies have suggested that PEMT is an important consumer of methyl groups from *S*-adenosylmethionine (SAM) and is a major determinant of homocysteine pools. Diabetes and all-*trans*-retinoic acid (ATRA) have been shown to alter the activities of several enzymes involved in methyl group metabolism. Thus, we investigated how diabetes and ATRA, individually and together, affect SAM-dependent phospholipid methylation. Rats received a single injection of streptozotocin (60 mg/kg body wt) or vehicle followed by administration of ATRA (30  $\mu$ mol/kg body wt) or vehicle for 5 d. The hepatic activity of PEMT increased 50% in both diabetic rat groups, whereas administration of ATRA was without effect. In diabetic rats, plasma total homocysteine decreased 30–35% in all treatment groups as compared with the control group. Thus, alterations in the activity of PEMT were not directly correlated to changes in homocysteine concentrations. Moreover, treatment of diabetic rats with insulin prevented the increase in PEMT activity and abundance. Because these observations support an increased need for SAM-dependent phosphatidylcholine synthesis, this may also indicate an increased choline requirement in diabetes. *J. Nutr.* 136: 3005–3009, 2006.

## Introduction

Phosphatidylcholine (PC)<sup>6</sup> is an important phospholipid component of cell membranes, hepatic lipoproteins, and bile and is often used in cell signaling pathways (1). Impaired PC biosynthesis can lead to an accumulation of triacylglycerols in the liver (2) and decreased secretion of VLDL (3), resulting in hepatosteatosis. PC can be synthesized by 2 different pathways. The CDP-choline pathway involves 3 different enzymes, choline kinase, CTP:phosphocholine cytidyltransferase, and choline-phosphate transferase, and is present in all nucleated cells (4,5). The second pathway, primarily present in the liver, is the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway. The PEMT pathway converts phosphatidylethanolamine

(PE) to PC by using *S*-adenosylmethionine (SAM) as a methyl donor with *S*-adenosylhomocysteine (SAH) as a by-product and accounts for 20–40% of normal hepatic PC synthesis (6).

Guanidinoacetate methyltransferase (GAMT), a hepatic enzyme that methylates guanidinoacetate to form creatine using SAM as the methyl donor, is the major consumer of methyl groups in the liver (7). It has been estimated that ~75% of the hepatic SAM pool is consumed by GAMT and ~15% by PEMT (7). However, a recently published paper (8) suggests PEMT consumes 2–3 times more of the hepatic SAM pool than GAMT and would therefore produce more homocysteine. The contribution of PEMT activity to homocysteine production is an important finding, because hyperhomocysteinemia has been shown to be associated with cardiovascular disease (9,10) and atherosclerosis (11). Thus, it is important to understand PEMT function, not only for its capacity to synthesize PC, but also for its role in homocysteine regulation.

Our laboratory has shown that diabetes and administration of pharmacological doses of all-*trans*-retinoic acid (ATRA) disrupts methyl group and homocysteine metabolism in the liver (12–15). The activity of glycine *N*-methyltransferase (GNMT), a key enzyme in the regulation of hepatic methyl group metabolism, was significantly increased following administration of streptozotocin (STZ) or ATRA (13). For diabetes, this induction of GNMT would be expected to decrease the methyl group pool and increase homocysteine concentrations; however, homocysteine

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<sup>6</sup> Abbreviations used: ATRA, all-*trans*-retinoic acid; BHMT, betaine homocysteine *S*-methyltransferase; GAMT, guanidinoacetate *N*-methyltransferase; GNMT, glycine *N*-methyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; STZ, streptozotocin; ZDF, Zucker diabetic (Type 2) fatty.

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concentrations were decreased because of an increase in the activity of the folate-independent enzyme betaine homocysteine S-methyltransferase (BHMT) and the abundance of cystathionine  $\beta$ -synthase. Because diabetes and ATRA disrupt the metabolism of methyl groups and homocysteine, it is important to determine their effects, if any, on SAM-dependent phospholipid methylation.

To date, studies directed at examining the relation between diabetes and PEMT have met with conflicting results (16–20). Hoffman et al. (17) and Cabrero et al. (16) reported that hepatic PEMT activity was decreased in alloxan-diabetic rats, which is similar to the decrease found in alloxan-diabetic sheep (19). For extrahepatic tissues, the activity of PEMT was increased in the cardiac subcellular membrane and brain of STZ-diabetic rats and mice, respectively (18,20). Although PEMT activity was not determined, Wijekoon et al. (21) recently reported a decrease in hepatic PC concentrations in Zucker diabetic (type 2) fatty (ZDF) rats, whereas plasma PC concentrations were elevated. It is not known how a diabetic state results in alterations of phospholipid methylation; however, it may be the result of characteristic changes in hormone levels such as insulin and glucagon (22–24). Therefore, it is important to determine how diabetes, alone and in combination with ATRA, affects phospholipid methylation and its potential influence on circulating levels of homocysteine.

## Materials and Methods

**Chemicals.** Reagents were obtained from the following: S-adenosyl-L-[methyl- $^3$ H]methionine, Perkin-Elmer Life and Analytical Sciences; porcine insulin, STZ, ATRA, and SAM, Sigma-Aldrich; polyclonal antibody directed against the C-terminal dodecapeptide of rat PEMT2 was raised in rabbits as previously described (25); goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase, Pierce. All other chemicals were analytical grade.

**Animals and diets.** All animal experiments were performed in compliance with the Iowa State University Laboratory Animal Resources Guidelines. For all studies, male Sprague-Dawley (Harlan Sprague-Dawley) rats (125–149 g) were housed in plastic cages with a 12-h light:dark cycle and consumed food (26) and water ad libitum. For Expt. 1, rats ( $n = 25$ ) were randomly assigned to receive a single intraperitoneal injection of STZ (60 mg/kg body wt) on d 0 and killed on d 3, 5, or 7. Nondiabetic control rats were injected with the vehicle (10 mmol/L citrate buffer, pH 4.5) and killed on d 0 and 7. For Expt. 2, rats ( $n = 24$ ) were randomly assigned to 1 of 4 treatment groups: control, STZ, ATRA, or STZ+ATRA. After diabetes initiation, ATRA was orally administered daily in corn oil (100  $\mu$ L/100 g body wt) at a dose of 30  $\mu$ mol/kg body wt for 5 d; control rats (nondiabetic and diabetic) received the same amount of corn oil alone. For Expt. 3, rats ( $n = 18$ ) were randomly assigned to 1 of 3 groups: control, STZ, or STZ with intraperitoneal insulin injections (1 unit/200  $\mu$ L saline, *bis in die*) for 5 d. For all studies, rats were anesthetized with an intraperitoneal injection of ketamine:xylazine (90:10 mg/kg body wt) and blood samples were collected via cardiac puncture in heparinized syringes; rats were killed by exsanguination. Samples of whole blood were used to determine blood glucose using a commercial kit (Sigma-Aldrich, kit 510). Additional whole blood samples were centrifuged at 4000  $\times$  g; 5 min and the plasma fraction was stored at  $-70^\circ\text{C}$  until analysis. Livers were rapidly removed and a 1-g portion was homogenized in 4 volumes of ice-cold phosphate-buffered (10 mmol/L, pH 7.0) sucrose (0.25 mol/L) containing 1 mmol/L EDTA, 1 mmol/L sodium azide, and 0.1 mmol/L phenylmethylsulfonyl fluoride. Following centrifugation at 20000  $\times$  g; 30 min, 1 mL of supernatant containing 1 mmol/L  $\beta$ -mercaptoethanol was stored at  $-70^\circ\text{C}$  for subsequent protein abundance measurements. Another 1-g portion was used for preparation of microsomes; remaining liver samples were snap-frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$ .

**Isolation of liver microsomes.** Approximately 1 g of liver was homogenized in 4 volumes of ice-cold 10 mmol/L Tris HCl (pH 7.4) containing 0.25 mol/L sucrose. The homogenate was centrifuged at 16000  $\times$  g; 20 min at  $4^\circ\text{C}$  followed by centrifugation of the supernatant (1–2 mL) at 105000  $\times$  g; 60 min at  $4^\circ\text{C}$  and the resulting pellet was resuspended in 400  $\mu$ L of 0.25 mol/L sucrose. The microsomal fraction was stored at  $-70^\circ\text{C}$  until determination of PEMT activity. The protein concentration of the microsomal fraction was determined by the Bradford method (27) using a commercial kit (Coomassie Plus, Pierce).

**PEMT activity and abundance.** The enzymatic activity of PEMT was determined by measuring the incorporation of radiolabeled methyl groups from S-adenosyl-L-[methyl- $^3$ H]methionine into phospholipids by the method of Duce et al. (28) with modifications. In brief, the reaction mixture (550  $\mu$ L) contained 0.1 mmol/L SAM, 2  $\mu$ Ci S-adenosyl-L-[methyl- $^3$ H]methionine, 10 mmol/L HEPES, pH 7.3, 4 mmol/L dithiothreitol, 5 mmol/L  $\text{MgCl}_2$ , and 750  $\mu$ g protein. Exogenous PE was not added to the reaction mixture, because it has not been shown to significantly increase the reaction rate (29,30).

For immunoblotting of hepatic PEMT2, 25  $\mu$ g of homogenate protein was separated on a 12.5% SDS-PAGE gel, transferred to nylon membranes, blocked with 5% milk, and probed with anti-PEMT2 antibody (diluted 1:5000 in Tween-Tris-buffered saline containing 5% milk) for 1 h. Membranes were then probed with goat anti-rabbit secondary antibodies (1:5000 in Tween-Tris-buffered saline + 1% milk) conjugated to horseradish peroxidase for 1 h and immunoreactive bands were visualized by enhanced chemiluminescence (Amersham) and quantified by densitometry (SigmaGel, SPSS).

**Plasma homocysteine.** Total homocysteine (free and protein bound) concentrations were determined by fluorometric HPLC (31). Samples (300  $\mu$ L plasma) were derivatized with 100  $\mu$ L of 4-fluoro-7-sulfo-benzofurazan (1 g/L) and 1 mmol/L N-acetylcysteine was added as an internal standard.

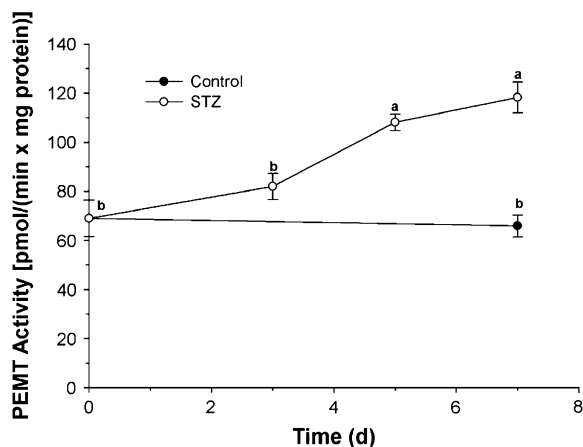
**Phospholipid analysis.** Total liver lipids were extracted (32) from frozen liver samples and fractionated on a silica Sep-Pak cartridge (Waters). Lipid extracts dissolved in chloroform:methanol (2:1) were loaded on the column and samples were sequentially eluted with 10 mL of chloroform for the neutral lipids, 15 mL of acetone:methanol (9:1) for the glycolipids and ceramides, and 15 mL of methanol for the phospholipids. The phospholipids in the methanol fraction were dried, reconstituted in 250  $\mu$ L chloroform, and a 20- $\mu$ L aliquot was injected onto an HPLC system for quantification of PE, PC, and total phospholipids using a Phospholipid Soy Lecithin Column (Astec) (33). Soy PE and PC standards (Avanti Polar Lipids) were used to quantify hepatic concentrations.

**Statistical analysis.** For Expts. 1 and 3, a 1-way ANOVA at the 5% significance level was performed. For Expt. 2, the means from each treatment group were compared using a 2-way ANOVA at a significance level of 5%. An ANOVA on ranks was performed when the test for normality failed. Means were compared using Fisher's least significant difference procedure. Correlation coefficients were determined using Pearson Product Moment. All statistical analyses were performed using SigmaStat 3.1 (SPSS).

## Results

**Hepatic PEMT activity was increased by diabetes.** In Expt. 1, the activity of PEMT increased over 7 d following the injection of STZ. PEMT activity was elevated 58% and 72% on d 5 and 7, respectively, compared with control values (Fig. 1). Circulating glucose concentrations were significantly elevated ( $\sim 25$  mmol/L) for all diabetic rats (3, 5, and 7 d) compared with both groups (0 and 7 d) of control rats ( $\sim 10$  mmol/L).

**Diabetes increased hepatic PEMT activity but reduced plasma total homocysteine concentrations.** Expt. 2 also



**Figure 1** In Expt. 1, STZ-mediated diabetes increased the activity of hepatic PEMT. Data are expressed as means  $\pm$  SEM,  $n = 5$ . Means without a common letter differ,  $P < 0.05$ .

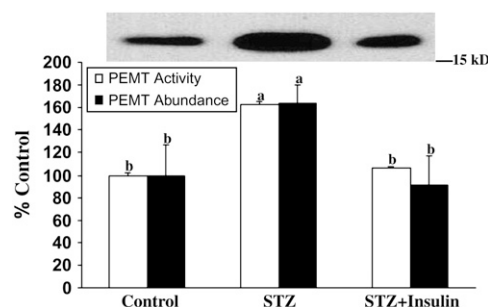
demonstrated that diabetes increased PEMT activity, with or without ATRA administration. All rats in the STZ and STZ+ATRA groups had blood glucose  $>20$  mmol/L ( $28.5 \pm 1.9$  and  $29.8 \pm 0.7$  mmol/L, respectively). Both groups of diabetic rats (STZ and STZ+ATRA) exhibited significantly higher PEMT activity after 5 d compared with control values (Table 1); however, administration of ATRA alone or in combination with STZ had no effect on PEMT activity. Despite the rise in PEMT activity, plasma homocysteine levels were reduced 32% in the STZ group compared with the control group. Similarly, plasma homocysteine concentrations were reduced  $\sim 35\%$  in the ATRA and the STZ+ATRA group. Therefore, no correlation was demonstrated between the activity of PEMT and circulating homocysteine concentrations for all 4 treatment groups ( $r = 0.109$ ,  $P = 0.611$ ) nor for the control and diabetic groups ( $r = -0.020$ ,  $P = 0.950$ ).

**Insulin injections prevented the rise in PEMT activity and abundance with diabetes.** For Expt. 3, the activity and abundance of PEMT were elevated in diabetic rats (Fig. 2). In contrast, PEMT activity and abundance in the STZ + insulin group was not significantly different from control values ( $P = 0.76$  and  $0.99$ , respectively). For all treatment groups, PEMT activity was correlated to PEMT2 protein abundance ( $r = 0.502$ ,  $P < 0.05$ ).

**TABLE 1** Hepatic PEMT activity and total plasma homocysteine concentrations from control, diabetic (STZ), ATRA, and STZ+ATRA treatment groups (Expt. 2)<sup>1</sup>

Treatment	PEMT activity <i>pmol · min<sup>-1</sup> · mg protein<sup>-2</sup></i>	Homocysteine $\mu\text{mol/L}$
Control	$38.9 \pm 5.6^a$	$16.7 \pm 0.8^a$
STZ	$58.2 \pm 5.3^b$	$11.3 \pm 1.6^b$
ATRA	$37.7 \pm 4.1^a$	$10.9 \pm 1.0^b$
STZ + ATRA	$60.4 \pm 7.1^b$	$10.1 \pm 1.1^b$
2-Way ANOVA <i>P</i> -values		
STZ	0.001	0.016
ATRA	0.927	0.007
STZ $\times$ ATRA	0.760	0.060

<sup>1</sup> Values are expressed as means  $\pm$  SEM,  $n = 6$ . Means within a column without a common letter differ,  $P < 0.05$ .



**Figure 2** Insulin treatment prevented the increase in PEMT activity and protein abundance in STZ-diabetic rats (Expt. 3). Data are expressed as means  $\pm$  SEM,  $n = 4-6$ . Means without a common letter differ,  $P < 0.05$ . PEMT activity is expressed as percent control with the control value being  $36.0 \pm 4.7$  pmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup>. A representative immunoblot is shown above the corresponding bars.

**Diabetes and ATRA administration decreased the hepatic PE:PC ratio.** Total hepatic PE concentrations and the percent PE of total phospholipids were reduced in the STZ and ATRA groups compared with control values (Table 2). Total hepatic PC concentrations were not significantly different among the treatment groups (data not shown); however, the percent PC of total phospholipids was increased in the STZ and ATRA groups. Collectively, these alterations in PE and PC concentrations reduced the ratio of PE:PC by 37% compared with control values.

## Discussion

Regulation of PEMT for hepatic SAM-dependent PC synthesis is an important transmethylation reaction and represents a significant use of methyl groups. We have shown in these studies that the activity and abundance of PEMT was elevated in a type 1 diabetes model, correlating with a decrease in the PE:PC ratio. Taken together with our previous finding that BHMT activity was elevated under diabetic conditions (13), these current studies may reflect an increased requirement for PC production from the SAM-dependent pathway. These results also may indicate that diabetes is characterized by an increased use of choline for homocysteine remethylation. It is not clear whether the increased production of PC is compensatory for the loss of

**TABLE 2** PE and PC compositions of rat livers from the control, diabetic (STZ), ATRA, and STZ+ATRA treatment groups (Expt. 2)<sup>1</sup>

Treatment	PE <i>% total phospholipids</i>	PC <i>% total phospholipids</i>	PE:PC
Control	$23.6 \pm 1.6^a$	$41.0 \pm 1.0^a$	$0.58 \pm 0.05^a$
STZ	$16.8 \pm 0.5^b$	$45.2 \pm 0.4^b$	$0.37 \pm 0.01^b$
ATRA	$16.6 \pm 0.5^b$	$45.2 \pm 0.4^b$	$0.37 \pm 0.01^b$
STZ + ATRA	$19.3 \pm 2.3^{a,b}$	$43.5 \pm 2.0^{a,b}$	$0.46 \pm 0.08^{a,b}$
2-Way ANOVA <i>P</i> -values			
STZ	0.242	0.148	0.281
ATRA	0.194	0.691	0.294
STZ $\times$ ATRA	0.011	0.011	0.011

<sup>1</sup> Values are expressed as means  $\pm$  SEM,  $n = 6$ . Means within column without a common letter differ,  $P < 0.05$ .

choline for homocysteine remethylation at the expense of CDP-dependent PC synthesis or is the result of a specific increased requirement for PC synthesis in diabetic rats, such as increased biliary secretion (34). There is evidence that PEMT activity can be regulated by the need for PC synthesis (35).

These results also have important implications for the relation between PEMT function and homocysteine homeostasis. Recent studies have shown that regulation of PEMT is directly correlated to homocysteine production and bile synthesis (36,37). Furthermore, PEMT activity is increased when the CDP-choline pathway is compromised, either by choline deficiency or by deletion of the gene encoding for CTP:phosphocholine cytidyltransferase- $\alpha$  (8). Moreover, the lethality due to hepatic depletion of PC in PEMT knockout mice may be the result of biliary secretion, and supplemental choline greatly enhances survival (38). Taken together with our findings, diabetes is a factor that has a major impact on the regulation of PEMT and PC synthesis, supported by the observation that biliary PC secretion was elevated in diabetic rats (34).

Of particular interest in our studies was the finding that, although PEMT activity was elevated in diabetic rats, it did not positively correlate with changes in homocysteine concentrations in the plasma, presumably because of the elevation in BHMT. Thus, in an acute diabetes model, the expected overproduction of homocysteine as a result of both PEMT and GNMT elevation appears to be compensated for by an increase in folate-independent homocysteine remethylation. This may be due in part to provide methyl groups for SAM-dependent transmethylation reactions, such as the synthesis of PC. Others have shown that increased catabolism of homocysteine via transsulfuration is also a major factor contributing to the hypohomocysteinemia exhibited by acute diabetic rats (39,40).

It is not clear as to the signal underlying regulation of PEMT in a diabetic state, although it is most likely the result of changes in hormone concentrations and concomitant alterations in the supply of substrates for methyl group/homocysteine metabolism. Incubating hepatocytes with glucagon or cAMP increases PEMT activity (23). Other factors that affect PEMT activity include vitamin B-6 status (41) and the ratio of SAM:SAH (42), although we did not find that methylation capacity (i.e. the ratio of SAM:SAH) was altered in diabetic rats (13). Other studies have found insulin treatment of STZ-diabetic rats prevents the increased abundance of BHMT and cystathionine  $\beta$ -synthase, as well as the decrease in plasma homocysteine concentrations (39,43). Similarly, we found insulin treatment of diabetic rats prevented the increase in PEMT activity and abundance. Because many of the findings reported for type 1 diabetes models have also been demonstrated in a type 2 model using ZDF rats (21), it is likely that changes in insulin production and/or tissue sensitivity is an important factor. In support of this, an insulin-sensitizing antidiabetic drug (Rosiglitazone) has been shown to decrease PEMT activity in some mouse models of type 2 diabetes (44).

Our previous studies using diabetic and ATRA-treated rats demonstrated a clear interaction between these 2 treatments with respect to folate-dependent and folate-independent remethylation of homocysteine, as well as its catabolism by the transsulfuration pathway (13). Moreover, the combination of diabetes or glucocorticoid treatment with ATRA administration exerted an additive effect on inducing GNMT expression (13,15). These findings suggest that ATRA administration alters methyl group and homocysteine metabolism in a manner that is distinct from diabetes and an interaction exists between them. Others have shown that vitamin A and its derivatives reduced SAM-dependent synthesis of PC and its concentrations in the

liver (45,46); however, in our studies reported here, no such interaction was evident with respect to the activity of PEMT and phospholipid methylation.

In summary, we have shown that type 1 diabetes is characterized by an increase in PEMT expression and by a state of hypohomocysteinemia, due to elevations in folate-independent remethylation of homocysteine. Choline is important for PC synthesis and is a considerable source of methyl groups in the liver following its oxidation to betaine. For ZDF rats, Wijekoon et al. (21) recently found reduced betaine concentrations in the liver and an increase in the activity of BHMT. Moreover, they also found decreased hepatic PC concentrations and attributed this to increased oxidation of choline to betaine for BHMT, thereby compromising the availability of choline for the CDP-pathway. Furthermore, PEMT activity was increased in mice fed a choline-deficient diet (35). Taken together with the increased utilization of PC for bile secretion, our research supports the possibility that diabetes may result in an increased choline requirement. This not only has implications for altered phospholipid methylation in diabetes, but also the subsequent regulation of homocysteine homeostasis. Thus, choline or betaine supplementation may be of benefit, because type 1 and type 2 diabetes progresses from hypohomocysteinemia to a state of hyperhomocysteinemia (21,47–52). The temporal and tissue-specific regulation of PEMT and homocysteine will be an important area for future research, as well as the potential benefit of choline and/or betaine supplementation.

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