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Influence of diabetes and retinoids on phospholipid methylation

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Influence of diabetes and retinoids on phospholipid methylation

by

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This is to certify that the master's thesis of

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has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

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LIST OF ABBREVIATIONS

5-CH_3-THF	5-methyltetrahydrofolate
5,10-CH_2-THF	5,10-methylenetetrahydrofolate
ATRA	all-*trans*-retinoic acid
BHMT	betaine-homocysteine *S*-methyltransferase
CBS	cystathionine β-synthase
CDP-choline	cytidine 5'-diphosphate choline
CT	CTP:phosphocholine cytidylyltransferease
DAG	diacylglycerol
GAMT	guanidinoacetate *N*-methyltransferase
GNMT	glycine *N*-methyltransferase
MS	methionine synthase
MTHFR	5,10-methylenetetrahydrofolate reductase
NASH	Non-alcoholic steatoheptatitis
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine *N*-methyltransferase
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine
STZ	streptozotocin
TAG	triacylglycerol
THF	tetrahydrofolate

ABSTRACT

Phosphatidylcholine (PC) is an essential compound for cell membranes, VLDL, cell signaling, and bile. PC can be synthesized directly from choline by the CDP-choline pathway or it can be synthesized via the methylation of phosphatidylethanolamine (PE) by phosphatidylethanolamine *N*-methyltransferase (PEMT). The latter of the two pathways requires methyl groups from three *S*-adenosylmethionines (SAM) and is responsible for ~30% of the hepatic PC production. Other methyltransferases, including betaine-homocysteine *S*-methyltransferase (BHMT) and glycine *N*-methyltransferase (GNMT), have been shown to have increased activity with diabetes. Furthermore, GNMT has increased activity with all-*trans*-retinoic acid (ATRA) supplementation. This thesis explores the affects of diabetes and ATRA supplementation on phospholipid methylation by PEMT. I found a 2-fold increase in PEMT activity with diabetes alone and in combination with ATRA supplementation. ATRA administration alone had no affect on PEMT activity. PEMT activity has been proposed to be an important regulator of homocysteine levels. However, we found a 30-35% decrease in homocysteine levels in diabetes when PEMT activity was elevated. There were decreased plasma total homocysteine concentrations with ATRA administration, which had no affect on PEMT activity. There was also a significant decrease in PE and an increase in PC as a percentage of total phospholipids in the diabetic and ATRA supplemented groups. I hypothesized the increased PEMT activity was the result of increased betaine usage by BHMT in diabetes, leading to an effective choline deficiency. Upon supplementation with choline or betaine in the diabetic state, there was no change in PEMT activity. These data suggest PEMT activity may be altered by hormonal changes in the diabetic state, rather than by a lack of choline or betaine.

CHAPTER 1. GENERAL INTRODUCTION

Thesis Organization

The first chapter of this thesis includes the organization of the thesis and a short description of the overall research questions addressed. Chapter 2 is a review of the relevant literature on methyl group metabolism, importance of homocysteine, phospholipid synthesis, and some potential modulators of these systems. Chapters 3 and 4 each focus on research conducted by the author. Chapter 5 provides general conclusions for the thesis. The Appendix presents research done by the author for a study conducted by Kristin Nieman. Chapters 3 and 4 and the appendix each contain an introduction, materials and methods, results, and discussion. Following the appendix is the comprehensive Literature Cited for all sections of this thesis.

Description of the Research Questions

Methyl groups from *S*-adenosylmethionine (SAM) can be transferred to a variety of compounds in the body including proteins, nucleic acids, and lipids. DNA methylation is required to maintain proper gene expression, while phospholipid methylation helps maintain cell membranes, cell signaling, and bile and VLDL secretion. A lack of methyl groups can lead to a variety of diseases including cancer, birth defects, and fatty liver (1-4). Furthermore, a lack of methyl groups can lead to elevated concentrations of homocysteine in the body, which is an independent risk factor for cardiovascular disease (4) and has been linked to a number of other diseases including Alzheimer's and osteoporosis (5).

Previously, my research group has shown Type 1 diabetes and administration of retinoids alters hepatic methyl group metabolism, both alone and in combination (6). In

some cases administration of retinoids was able to attenuate the effects of diabetes on methyl group metabolism.

Phosphatidylcholine (PC) synthesis via methylation by phosphatidylethanolamine *N*-methyltransferase (PEMT) is responsible for ~30% of the PC produced by the liver (7-9). This reaction requires the use of three methyl groups from SAM and consequently produces three *S*-adenosylhomocysteines (SAHs). The potentially high usage of methyl groups by PEMT has led many researchers to believe PEMT is an important regulator of homocysteine concentrations in the body (10). Therefore, it is important to determine how factors that affect methyl group metabolism and plasma homocysteine levels, e.g., diabetes and retinoid administration, affect phospholipid methylation. Furthermore, if PEMT activity is affected, what consequences does this altered activity have on liver lipid and phospholipid concentrations. This thesis examines how phospholipid methylation is affected by diabetes and retinoids, what consequences altered phospholipid methylation has, and explores nutrient supplementation to correct changes in phospholipid methylation.

CHAPTER 2. LITERATURE REVIEW

Methyl Group Donors

Choline

Approximately 30 mmoles of methyl groups/d are consumed as choline in the diet, and it is estimated over 60% of the free choline in the liver is converted to betaine (11). The dietary reference intake (DRI), established by the Food and Nutrition Board of the Institute of Medicine 1997-2001, for choline is 425 mg/d and 550 mg/d for adult women and men, respectively. The upper intake limit for adults is 3.5 g/d. Plants and animals alike have the ability to synthesize choline. Choline is consumed as free choline or lecithin. Liver, eggs, soybeans, and peanuts (12, 13) are good sources of lecithin, which is the major form of choline consumed in the human diet (14). Choline concentrations in blood and tissues are directly proportional to the amount of choline consumed in the diet (15). Much of the choline consumed in the diet is rapidly "trapped" by the liver, but some is removed by the kidney, oxidized to betaine, and excreted (14). The liver and the kidney are important in maintaining choline concentrations in the plasma. Choline is an important dietary component because it is the precursor for sphingomyelin, phosphatidylcholine (PC), acetylcholine, platelet-activating factor (PAF), and betaine (16) (**Figure 2.1**).

Choline Supplementation

Choline is used in the treatment of several diseases. Choline is converted to acetylcholine by choline acetyltransferase (14). Systemic administration of pharmacological doses of choline rapidly increases the concentration of choline in the brain (17, 18). Increased choline concentrations, in turn, leads to increased acetylcholine concentrations in

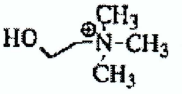
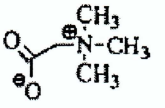
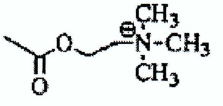
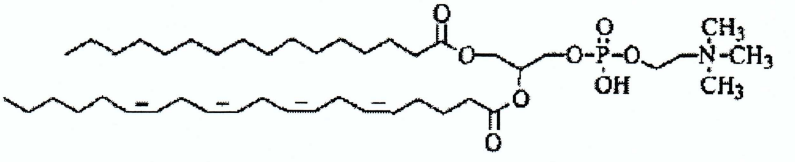
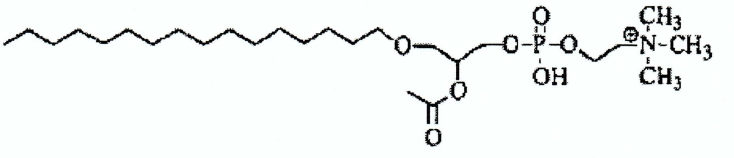
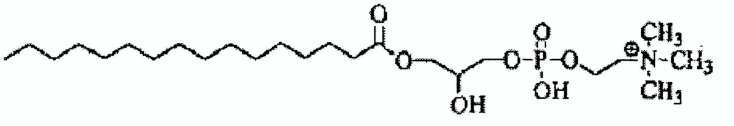
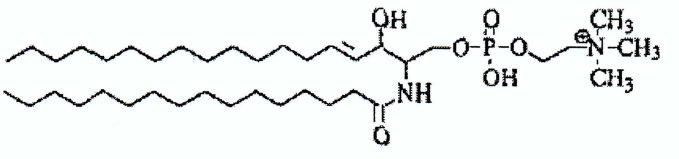
Compound	Chemical structure
Choline	
Betaine	
Acetylcholine	
Phosphatidylcholine	
Platelet-activating factor	
Lysophosphatidylcholine	
Sphingomyelin	

Figure 2.1. Structure of choline and selected metabolites (modified from Zeisel and Blusztajn, 1994)

the brain, peripheral cholinergic neurons, and tissues. Pharmacological doses of choline have been used to treat tardive dyskinesia (19), a movement disorder, and improve memory (20), which may have implications in treating Alzheimer's disease (21).

Choline supplementation also has been used in the treatment of hypertension (14). Choline administration may lower blood pressure through the cholinergic vagus nerve, through the vasodilator effects of choline, or a combination of both (22). Another possibility is increased synthesis of PAF, which has the ability to lower blood pressure (16).

Additionally, choline has been shown to affect plasma lipids, but with conflicting results. Oral choline supplementation increased plasma cholesterol, phospholipid, HDL, and LDL concentrations (23, 24). However, there have been studies showing a lowering of blood cholesterol with choline supplementation (16). With such inconsistent results, choline administration is not the optimal choice for treatment of hypercholesterolemia.

Choline deficiency

Choline deficiency, though rare in humans, has been studied extensively in animals. A choline-deficient diet can rapidly, within hours or days in rats, cause an accumulation of lipids in the liver, particularly triacylglycerols (TAGs) (25, 26). This may be due to a decreased ability to assemble lipoproteins in order to transport TAGs out of the liver. This view is further supported by the fact that choline deficiency also decreases blood VLDL concentrations. Because of the intimate involvement of phosphatidylcholine in the synthesis and secretion of lipoproteins, a decrease in liver phosphatidylcholine could alter these processes.

Fatty liver is not the only health consequence of choline deficiency. A choline-deficient diet causes kidney lesions in weanling rats (25). Also, choline deficiency causes

irreversible hypertension in rats (27). Furthermore, choline-deficiency is the only known nutrient deficiency that can cause spontaneous hepatocarcinomas in the absence of a carcinogen (16). Additionally, choline deficient rats are more sensitive to cancer causing agents.

Betaine

Betaine has a chemical formula of $(CH_3)_3N^+CH_2COO^-$ and a molecular weight of 117.2 g/mol (1). Average dietary betaine intakes range from 1 g/d to as much as 2.5 g/d. However, there have been studies that supplemented up to 20 g/d of betaine for one year and that dosage was well tolerated (28). Betaine is found in many foods including wheat, shellfish, spinach, and sugar beets. Betaine accumulates in plants as a result of drought, high salinity, or temperature stress. In addition to obtaining betaine directly from the diet, choline can also serve as a source of betaine. Choline can be oxidized to betaine aldehyde by the enzyme choline dehydrogenase in mitochondria and betaine aldehyde is oxidized to betaine by betaine aldehyde dehydrogenase in mitochondria and cytosol. Betaine donates one methyl group to homocysteine to form methionine and dimethylglycine using the enzyme betaine-homocysteine *S*-methyltransferase (BHMT). Dimethylglcyine donates another methyl group to tetrahydrofolate to form sarcosine and 5,10-methylenetetrahydrofolate in a reaction catalyzed by dimethylglycine dehydrogenase (29). Sarcosine is further demethylated by sarcosine dehydrogenase to form glycine and 5,10-methylenetetrahydrofolate. In all, betaine can donate three methyl groups to the one carbon pool, either directly or via re-methylation of tetrahydrofolate.

In patients with homocystinuria, which is marked by elevated plasma homocysteine concentrations, oral betaine supplementation was able to effectively decrease plasma total

homocysteine (29). Oral betaine supplementation decreases plasma total homocysteine in healthy human subjects in a dose-dependent manner (30). Studies have shown dietary supplementation of choline in rats increases hepatic betaine concentrations regardless of the amount of methionine in the diet (29). This increase in liver betaine concentrations is accompanied by an increase in BHMT concentrations (31).

Other than donating methyl groups, betaine also plays a role as an organic osmolyte (29). Cells maintain their hydration state by altering concentrations of intracellular inorganic ions, such as sodium and potassium, and organic osmolytes, such as betaine (1). Inorganic ions can only accumulate to a certain level due to their effects on protein structure and function. Betaine, however, has very little interaction with protein surfaces, and so betaine can accumulate to high levels in the cell to maintain osmolarity without affecting normal cell function.

Folate

Folate is a general term that actually describes many compounds derived from tetrahydrofolate (THF) (32). Dietary folate contributes approximately 5-10 mmoles methyl groups/d (3). Folate, in the form of 5-methyltetrahydrofolate (5-CH_3-THF), donates one methyl group to homocysteine to form methionine and THF. This reaction is catalyzed by methionine synthase (MS), a B_{12} dependent enzyme. THF can be converted to 5,10-methylenetetrahydrofolate (5,10-CH_2-THF), which can then be converted to 5-CH_3-THF by 5,10-methylenetetrahyrofolate reductase (MTHFR). In 1998, the Food and Drug Administration (FDA) required that all enriched grain products be fortified with folate. Folate fortification led to a decreased prevalence of folate-deficient related diseases (33). In particular, the incidence of the neural tube defect spina bifida has decreased by 20% in the

United States (2).

Methionine

Methionine is a sulfur-containing essential amino acid. Methionine is converted to the active form, *S*-adenosylmethionine (SAM), by the enzyme methionine adenosyltransferase. Methionine is one of the primary sources of methyl groups in human foods, with a consumption of approximately 10 mmol methyl groups/d (3). Homocysteine can be converted to methionine using methyl groups from betaine or 5-CH_3-THF (34). Deficiency of methionine leads to a decrease in the SAM:SAH ratio (35). Pyridoxine (B_6) deficiency leads to similar results, probably because it is a cofactor for cystathionine β-synthase (CBS). Pyridoxine deficiency inhibits the activity of CBS, leading to decreased catabolism of homocysteine by the transsulfuration pathway. Decreased catabolism of homocysteine can result in a "build up" of homocysteine, which is in equilibrium with *S*-adenosylhomocysteine (SAH), and it is the increased SAH concentrations that inhibit methyltransferase activity.

Methyl Group Metabolism

Methyl group metabolism involves the activation of methionine being converted to its active form SAM, which then donates a one-carbon group to various biomolecules including proteins, nucleic acids, and phospholipids (**Figure 2.2**). This reaction leaves SAH which is then hydrolyzed to homocysteine. Homocysteine can be re-methylated to methionine, it can be converted to cysteine, or it can be secreted from the cell. Betaine and 5-CH_3-THF are the methyl group donors for re-methylation of homocysteine. Regulation of this pathway is important to maintaining the normal cell cycle. In the following sections, each part of this pathway will be discussed in detail.

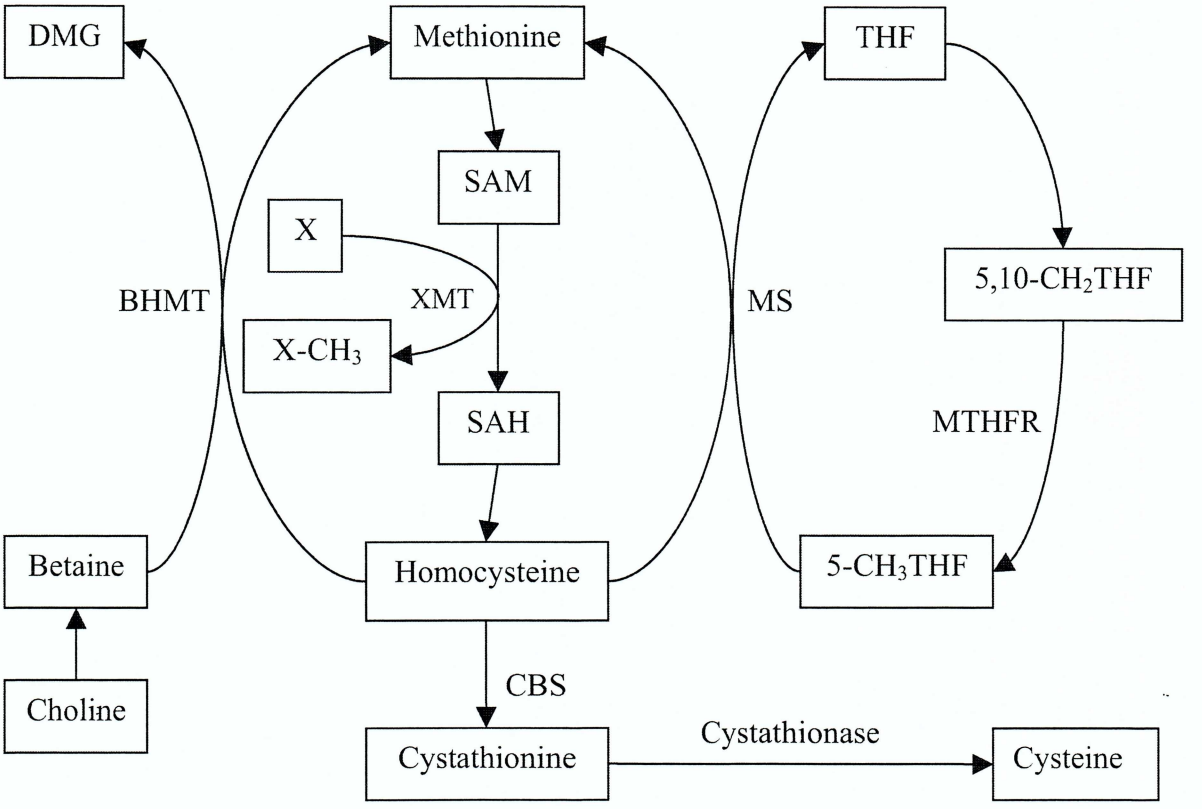


Figure 2.2. Methyl group metabolism pathway
BHMT = Betaine-homocysteine *S*-methyltransferase; CBS = Cystathionine β-synthase; DMG = Dimethylglycine; 5-CH_3-THF = 5-Methyltetrahydrofolate; 5,10-CH_2-THF = 5,10-Methylenetetrahydrofolate; SAM = *S*-adenosylmethionine; SAH = *S*-adenosylhomocysteine; THF = Tetrahydrofolate; XMT = methyltransferase

Transmethylation

Methyltransferases (shown as XMT in Figure 2.2) catalyze the removal of a methyl group from SAM to form SAH. In this reaction, the methyl group from SAM is donated to a variety of molecules including protein, lipids, and nucleic acids. Most methyltransferases are located predominantly in the liver, pancreas, and kidney. There are many methyltransferases in animals, but this review will focus on the three primary users of SAM in the liver, glycine *N*-methyltransferase (GNMT), guanidinoacetate *N*-methyltransferase (GAMT), and

phosphatidylethanolamine *N*-methyltransferase (PEMT).

Glycine *N*-methyltransferase

Glycine *N*-methyltransferase is located in the liver, pancreas, and kidney and comprises 0.9-3% of all the hepatic cytosolic protein (36). This enzyme is responsible for ~20% of the SAM-dependent one carbon reactions in rabbit liver, but this value varies among species (36). GNMT catalyzes the addition of a methyl group from SAM to glycine to create sarcosine. Unlike many other methyltransferases, GNMT is not as sensitive to feedback inhibition by SAH. GNMT is allosterically inhibited by 5-CH_3-THF, an intermediate in the folate metabolic pathway (37, 38). The inhibition of GNMT activity is a key component that enables GNMT to optimize the SAM to SAH ratio in the cell, thereby increasing the effectiveness of other methyltransferases.

Guanidinoacetate *N*-methyltransferase

Guanidinoacetate is produced by the kidney and travels to the liver via the blood. There, it is converted to creatine by the enzyme GAMT, which converts one SAM to SAH in the process. GAMT is expressed predominantly in the liver and pancreas, with expression in other tissues being too low for detection (39). In healthy humans, this reaction produces ~1 g/d of creatine, but can be decreased by creatine supplementation (40). Reduced creatine synthesis is due to the feedback inhibition of creatine on GAMT. Therefore, endogenous creatine production returns to normal levels upon termination of supplementation (41). GAMT deficiency is an autosomal recessive genetic disorder that results in under production of creatine (39). This lack of creatine biosynthesis leads to epilepsy, muscular hypotonia, and mental retardation.

According to Mudd *et al.* (42), GAMT uses approximately 75% of the hepatic one-

carbon pool, making it the largest consumer of SAM in the liver. However, Jacobs *et al.* (43) recently presented evidence that phosphatidylethanolamine *N*-methyltransferase (PEMT) may consume more SAM than GAMT. This finding is important because the methyltransferase that consumes the most SAM can have a large impact on other methyltransferase reactions and homocysteine concentrations. In a study published by Stead *et al.* (44), rats were fed a diet supplemented with guanidinoacetate or creatine for two weeks. Plasma total homocysteine was increased by 50% in rats supplemented with guanidinoacetate whereas plasma total homocysteine was decreased by 25% in rats supplemented with creatine. The results of the study by Stead *et al.* further elucidate the role of GAMT activity in regulating plasma homocysteine levels.

Phosphatidylethanolamine *N*-methyltransferase

As the primary focus of this research is on PEMT, this enzyme will be discussed in depth in a later section.

Re-methylation

Re-methylation of homocysteine to methionine conserves the one-carbon skeleton (45). Re-methylation can be achieved by one of two enzymes, betaine-homocysteine *S*-methyltransferase or methionine synthase. Methionine levels often depend on the ability of these two enzymes to function properly.

Betaine-homocysteine *S*-methyltransferase

Betaine-homocysteine *S*-methyltransferase is one of two enzymes capable of re-methylating homocysteine to methionine. BHMT is a folate-independent enzyme that catalyzes the conversion of betaine to dimethylglycine (DMG) by donating a methyl group from betaine to homocysteine to form methionine. The activity of BHMT is elevated when

betaine is supplemented in the diet. BHMT is predominately located in the liver; however, in some species, such as humans and pigs, there is appreciable BHMT activity in the kidney (46).

Methionine synthase

Methionine synthase (MS) is a folate-dependent enzyme with a cobalamin (B_{12}) cofactor. MS is responsible for the re-methylation of homocysteine to methionine using 5-CH_3-THF as a methyl donor. Unlike other enzymes involved in methyl group metabolism, MS is ubiquitously expressed in most tissues (46). MTHFR is responsible for irreversibly converting 5,10-CH_2-THF to 5-CH_3-THF and is also universally expressed in most tissues. Because MTHFR is unidirectional under physiological conditions, if there is a cobalamin deficiency and 5-CH_3-THF can not be converted to THF, then there is a "build-up" of 5-CH_3-THF. MTHFR is sensitive to allosteric regulation and is inhibited by elevated levels of SAM.

Transsulfuration

Transsulfuration is vital to regulation of homocysteine levels. The enzymes of the transsulfuration pathway, cystathionine β-synthase (CBS) and cystathionine γ-lyase (cystathionase), are predominately located in the liver, pancreas, and kidney. These enzymes are especially important in the kidney. In a study by House *et al.* (46), they found ~80% of plasma homocysteine was removed by the kidney in rats, with little to no urinary excretion of homocysteine. Furthermore, they found there was little re-methylation of homocysteine to methionine in the kidney; so the ability of the kidney to regulate plasma homocysteine concentrations was primarily a result of the transsulfuration pathway.

Cystathionine β-synthase

Cystathionine β-synthase is the first enzyme in the transsulfuration pathway of homocysteine. CBS irreversibly condenses homocysteine and serine to form cystathionine. CBS has a pyridoxine requirement for activity. There are polymorphisms of CBS that inhibit the activity of the transsulfuration pathway, but about half of the subjects are responsive to supplementation with large doses of pyridoxine (47). Inborn errors of CBS can lead to homocystinuria (48). CBS can be activated by elevated levels of SAM and SAH (46).

Cystathionine γ-lyase

Cystathionine γ-lyase (cystathionase) is the next enzyme in the transsulfuration pathway, and it also has pyridoxine as a cofactor. Cystathionase converts cystathionine to cysteine, α-ketobutyrate, and NH_4^+ (46). The cysteine can be used for glutathione synthesis, an important compound in redox reactions. Cysteine can also be used for protein synthesis, or it can be further catabolized.

Homocysteine

Biochemistry of homocysteine

Homocysteine is a sulfur-containing amino acid and is present in the body in the L-homocysteine form (49). Most of the homocysteine present in the body is from catabolism of methionine, with very little intake from the diet (50). *S*-adenosylhomocysteine is converted to homocysteine in a reversible reaction catalyzed by *S*-adenosylhomocysteine hydrolase. This enzyme is present in a wide variety of tissues with the most activity in pancreas, liver, and kidney tissues. The equilibrium of this reaction favors SAH; however, *in vivo* homocysteine is rapidly converted to methionine or cystathionine to ensure that the reaction favors the formation of homocysteine (49).

Homocysteine exists in the plasma as ~30% free and ~70% protein-bound, mostly to albumin (50). Homocysteine binds albumin through the formation of disulfide bonds with cysteine residues. Less homocysteine is protein-bound in the cytosol of tissues, with anywhere from 5-40% being protein-bound in various tissues of the rat (51). There is little known about homocysteine concentrations in human tissues, so it is not known if plasma concentrations reflect tissue concentrations (50).

Diseases associated with homocysteine

Homocystinuria is a disease in which homocysteine concentrations in plasma are severely elevated causing urinary excretion of homocysteine (48). The first case was reported in 1962, followed by rapid reporting of more cases. Homocystinuria is a result of inborn errors of CBS or MS. Complications of homocystinuria include dislocation of the ocular lens, skeletal abnormalities, mental retardation, and vascular disease. Treatments for homocystinuria have focused on lowering plasma homocysteine concentrations. As mentioned previously, about half of the subjects with CBS deficiency are able to lower their plasma homocysteine concentrations with pyridoxine supplementation. Treatments for those who are unresponsive to pyridoxine supplementation or have a defect in MS include a low methionine diet and betaine supplementation. Lowering of plasma total homocysteine in these subjects reduces their risk for cardiovascular disease.

The complications of homocystinuria led researchers to study the impacts of mildly elevated plasma total homocysteine concentrations, or hyperhomocysteinemia. Hyperhomocysteinemia is characterized by plasma total homocysteine concentrations greater than 15 μM, with normal levels ranging from 5-15 μM (46). Hyperhomocysteinemia is considered an independent risk factor for cardiovascular disease (4, 52) and atherosclerosis

(53). It is not necessary to have extremely high levels of homocysteine to be at increased risk. There is a 60% and 80% increased risk of coronary heart disease in women and men, respectively, with just a 5 μM increment in total homocysteine (54).

The reduction in neural tube defects with folate supplementation indicated that elevated homocysteine concentrations may play a role in their development. Birth defects are the leading cause of infant mortality, causing 22% of all infant deaths (2). Seven percent of these deaths are caused by neural tube defects. A study by Rosenquist *et al.* (55) found that treating chick embryos with homocysteine caused neural tube defects and ventricular septal defects despite normal levels of folate. Other pregnancy complications believed to be associated with hyperhomocysteinemia include spontaneous abortion and early-onset preeclampsia (56).

Finally, patients with Alzheimer's disease commonly have elevated levels of homocysteine and low circulating levels of folate and cobalamin (57). These observations seem to be consistent with the fact that subjects with homocystinuria commonly experience mental retardation. Taken together, these findings could mean that folate supplementation could prevent or delay the onset of Alzheimer's disease, which is significant because at this time there is no cure for Alzheimer's disease.

It is important to remember that most of the studies showing elevated homocysteine levels to be associated with certain diseases are epidemiological studies and therefore can not show a cause and effect relationship. Homocysteine may just be one sign of other disorders that are actually causing the disease. This means that it may not be enough to lower homocysteine levels to prevent disease; it may be necessary to treat the underlying cause of the elevated homocysteine levels to prevent disease. More research needs to be done to

elucidate the relationship between hyperhomocysteinemia and health complications.

Phosphatidylcholine

Synthesis

CDP-choline pathway

Phosphatidylcholine (PC) can be synthesized by the CDP-choline pathway, also

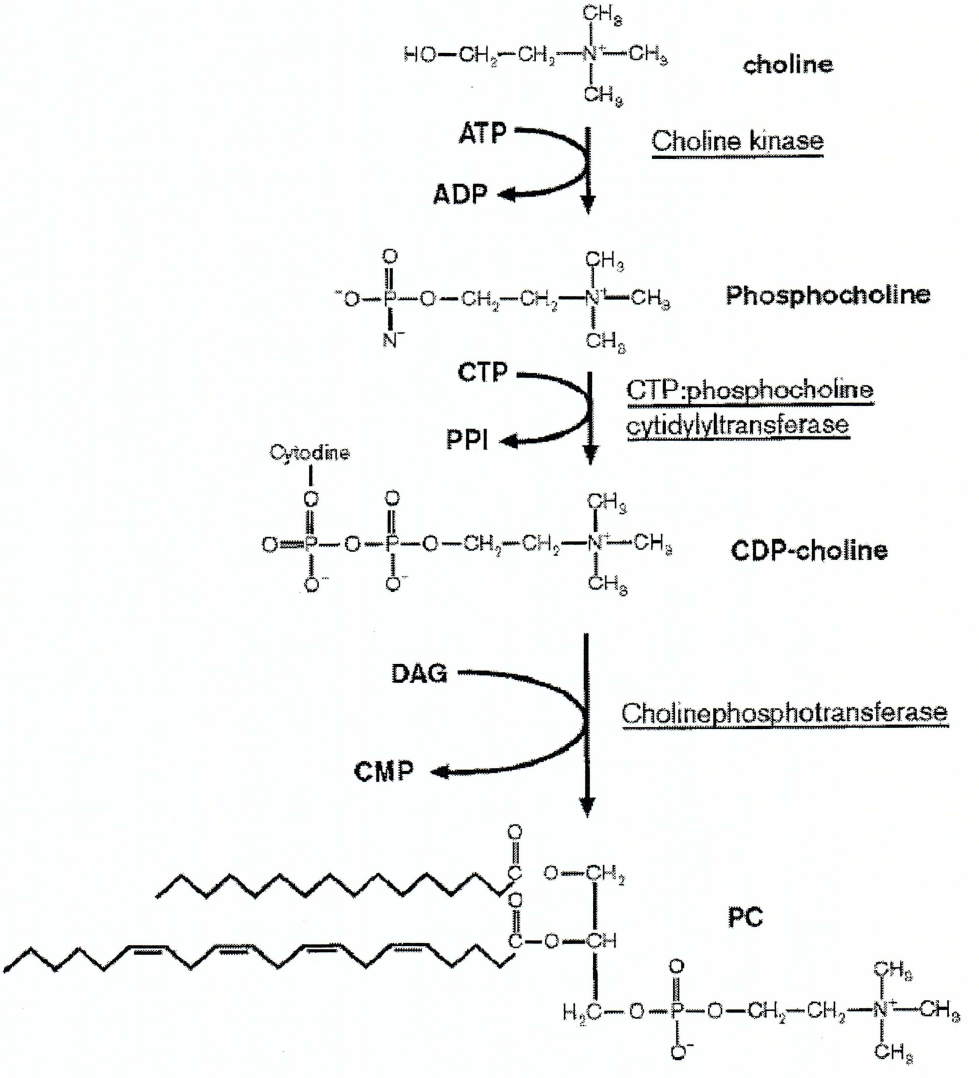


Figure 2.3. Phosphatidylcholine synthesis via CDP-choline pathway (modified from Vance and Walkey, 1998)

known as the Kennedy pathway (**Figure 2.3**). It is estimated that 70% of hepatic PC is synthesized by the CDP-choline pathway (58). Choline is converted to phosphocholine by choline kinase using ATP as a phosphate donor. Phosphocholine is then combined with cytidine triphosphate (CTP) to form CDP-choline by CTP:phosphocholine cytidylyltransferase (CT). This is the rate-limiting step in the CDP-choline pathway. CDP-choline is then combined with diacylglycerol (DAG) catalyzed by phosphatidylcholine glyceride transferase to form PC. The CDP-choline pathway mainly synthesizes medium chain and saturated PC (7).

PC can be catabolized to phosphatidic acid (PtdOH) and choline by phospholipase D (PLD) (**Figure 2.4**) (59). In this manner, the PC pool can serve as a store of choline. Furthermore, synthesis of PC by the methylation pathway can be considered *de novo* synthesis of choline. The phosphatidic acid (PtdOH) produced by this enzyme can be broken down further by phospholipase A_2 (PLA_2) to lysophosphatidic acid, which is used in cell signaling as described later.

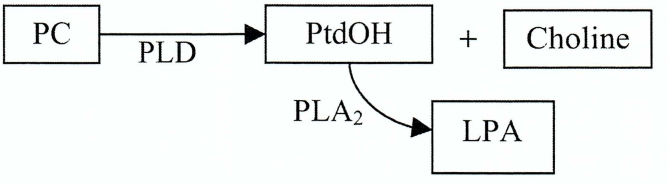


Figure 2.4. Catabolism of PC to choline

PEMT pathway

An alternative pathway for PC production in the liver is via methylation of phosphatidylethanolamine (PE) by phosphatidylethanolamine *N*-methyltransferase (PEMT). PEMT is a methyltransferase that transfers methyl groups from three SAMs to PE to form three SAHs and one PC (**Figure 2.5**). The intermediates of this reaction are

phosphatidylmonomethylethanolamine (PMME) and phosphatidyldimethylethanolamine (PDME).

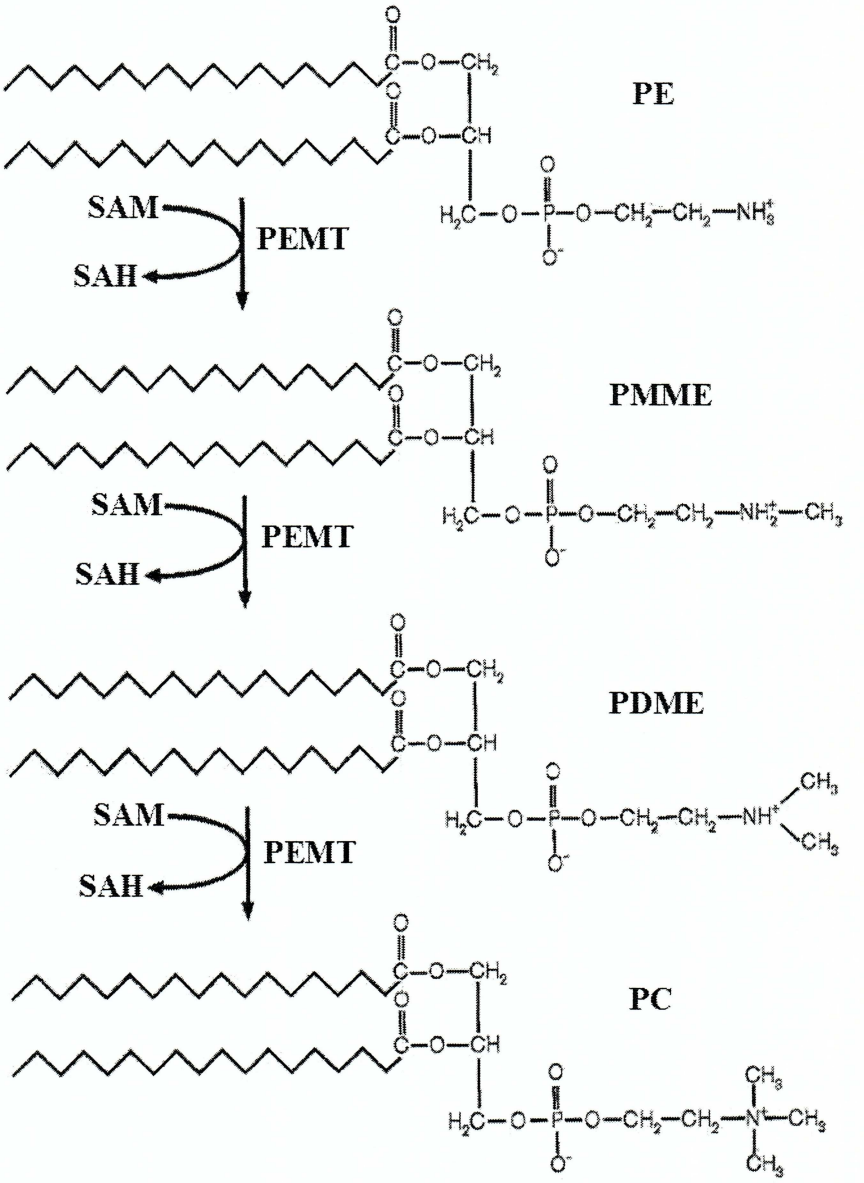


Figure 2.5. Methylation of phosphatidylethanolamine to phosphatidylcholine (modified from Vance and Walkey, 1998)

PEMT is a 22.5-kDa protein that is mainly associated with the endoplasmic reticulum, but a second form of PEMT can be found on the mitochondria-associated

membrane (60). The two forms are encoded by the same gene and deletion of this gene removes all hepatic PEMT activity. PEMT is considered a liver specific enzyme, but there have been trace amounts found in other tissues (**Table 2.1**). PEMT is very active in the myelinating fetal brain, with activity becoming almost nonexistent after birth (61).

Tissue	Subcellular fraction	Specific activity (pmol/min/mg)	pH	AdoMet concentration	Additional cofactors
Rat liver	microsomes	450	9.2	200	—
		580	9.2	200	0.28 mg/ml PMME
		550	9.2	200	0.85 mg/ml PDME
Mouse liver	microsomes	209	8.8	24	
		226	8.8	24	0.6 mM PE
		673	8.8	24	0.6 mM PMME
		463	8.8	24	0.6 mM PDME
Rat liver	microsomes	31	8.0	200	—
Rat heart	homogenate	4.3–9.6	9.0	200	10 mM $MgCl_2$
Rat heart	sarcolemma	4.3	10	150	—
	microsomes	4.6	10	150	—
	mitochondria	4.4	10	150	—
Hamster heart	sarcolemma	0.1	6	0.055	1 mM $MgCl_2$
	sarcolemma	0.05	7	10	1 mM $MgCl_2$
	sarcolemma	3.60	10	150	1 mM $MgCl_2$
Rat colonic epithelial cells	plasma membranes	3.66	8.0	200	—
Rat adipocytes	plasma membranes	5.0	9.2	10	0.1 mM PMME
Rat adipocytes	plasma membranes	4.8	8.5	10	0.1 mM PMME
Rat erythrocytes	plasma membranes	0.49	8.0	200	5 mM $MgCl_2$
Human neutrophils	microsomes	0.6	8.0	50	—
Human lymphocytes	microsomes	0.84	8.0	50	—
Rat aorta	microsomes	0.42	8.2	100	5 mM $MgCl_2$
Mouse thymus	microsomes	0.01	7.4	20	0.4 mg/ml PE
		0.02	7.4	20	1.0 mg/ml PMME
		0.06	7.4	20	1.0 mg/ml PDME
Bovine adrenal medulla	microsomes	3.87	7.0	4.0	5 mM $MgCl_2$
Whole rat brain	microsomes	0.91	8	200	1 mM $MgCl_2$
	synaptosomal plasma membrane	0.93	8	200	1 mM $MgCl_2$
Rat pituitary	homogenate	0.21	9.5	200	10 mM $MgCl_2$
	homogenate	0.06	6.5	200	10 mM $MgCl_2$
		0.11	9.5	200	10 mM $MgCl_2$
Dog lung	microsomes	0.78	8.2	200	6.6 mM $MgCl_2$; 0.3 mM dog lung PE

Table 2.1. Distribution of PEMT activity among various tissues (modified from Vance and Ridgeway, 1988)

Conversely, PEMT is not significantly expressed in the liver until one day before birth (62). However, PEMT expression can be decreased by partial hepatectomy (63). PEMT activity

decreased by 50% within 24 hr of partial hepatectomy, with activity returning to normal by the time liver reached adult size. Additionally, when lead nitrate was used to stimulate liver growth, there was a decrease in PEMT activity (64). This evidence suggests that PEMT is less active in rapidly dividing liver cells. This idea led researchers to investigate the role of PEMT in liver cell growth. PEMT activity is practically undetectable in hepatocarcinoma cell lines and transfection of PEMT into hepatocarcinoma cells such as McArdle RH-7777 cells results in overexpression of PEMT and inhibition of cell growth (65). The authors believe PEMT is involved in signaling for differentiation and apoptosis.

The PEMT pathway accounts for approximately 30% of the PC production in the liver (7-9). In contrast, PC synthesis by the PEMT pathway is not necessary for survival, as *Pemt*$^{-/-}$ knock out mice can live long lives when maintained with a choline-sufficient diet (66). However, when *Pemt*$^{-/-}$ knock out mice are fed a diet deficient in choline, they die within a few days. Times of choline deficiency may be one reason for the conservation of PEMT throughout evolution. PEMT synthesizes mostly long chain and highly unsaturated PC species (18:1/18:1; 18:0/18:2; 18:2/20:4; 18:1/20:4; 18:0/20:4; 18:0/22:6; 18:1/22:5) as opposed to the CDP-choline pathway, which mostly forms medium chain and saturated PC species (16:0/18:1; 18:0/18:2; 18:1/18:1) (7). PC synthesized by the two pathways may be utilized in different ways in the cell, such as PC from PEMT being targeted to VLDL synthesis (67). Differences in length and saturation of PC formed by the two pathways gives another possible reason why PEMT is physiologically significant.

It was previously thought that PEMT consumed approximately 15% of the SAM pool, whereas GAMT, another methyltransferase, used about 75% of the pool (42). As mentioned previously, this belief has been recently questioned in a paper by Jacobs *et al.* (43)

that indicated PEMT consumed 2-3 times more SAM than GAMT and would therefore be a greater contributor to SAH production. These findings are logical, as PEMT consumes three SAMs to make one PC, while GAMT only consumes one SAM to make creatine. If PEMT is a major producer of homocysteine, then PEMT may also play an important role in the regulation of homocysteine. In CTα-deficient mice hepatocytes, there is an 80% increase in PC synthesis via PE methylation and a 40% increase homocysteine secretion (43). Additionally, *Pemt*$^{-/-}$ knockout mice have 50% lower plasma homocysteine concentrations and hepatocytes isolated from these mice secrete 50% less homocysteine (10). Furthermore, cell lines stably expressing various levels of ER- and MAM-localized PEMT showed increased homocysteine secretion with increased PEMT activity (68).

PEMT activity has been shown to be regulated by a number of things including substrate, product, and hormones. Similar to other methyltransferases mentioned earlier in the review, the concentration of SAH seems to play a role in regulation of PEMT activity because SAH has a higher affinity for the binding site of PEMT than for SAM (60, 69). In a study by Hoffman *et al.*(70), a decrease in the SAM:SAH was responsible for drastically reducing the activity of PEMT. Similarly, rats fed a diet deficient in pyridoxine, which is a necessary cofactor for catabolism of homocysteine by the transsulfuration pathway, had significantly less PC synthesized by the methylation pathway as compared with controls (35). The authors believed an increase in SAH was responsible for the decreased PEMT activity. PE concentrations also seem to be important regulators of PEMT activity, but PC has yet to be shown to affect PEMT activity (60).

Availability of choline in the diet can also affect PEMT activity. Rats fed a choline-deficient diet experience a 63% increase in PEMT activity within two days (71), but they do

not experience an increased expression of PEMT until they are on a choline deficient diet for more than three weeks (58).

Castaño *et al.* (72) showed incubation of rat hepatocytes with glucagon increased the activity of PEMT. Incubation of rat hepatocytes with cAMP or NaF, an activator of adenylate cyclase, had similar effects as glucagon. Additionally, incubation with a phosphodiesterase inhibitor, which decreases intracellular cAMP, significantly decreased the activity of PEMT. This inhibition is strong evidence that PEMT activity can be hormonally regulated, possibly via changes in the phosphorylation state of the enzyme or induction of gene expression.

Coordination of the two pathways

There is mounting evidence that PC synthesis via the CDP-pathway and the PEMT pathway is coordinated. When 3-deazaadenosine was injected into rats, it increased the concentration of SAH in the liver, which effectively inhibited the activity of hepatic PEMT (73). There was a concurrent increase in PC synthesis by the CDP-choline pathway. Hepatocytes isolated from CTα-deficient mice have CT activity that is only 15% of control values, with only a 10-20% decrease in hepatic PC levels, suggesting increased PC synthesis via PE methylation (43). Similarly, when McArdle RH-7777 rat hepatoma cells, which lack PEMT expression, are transfected with cDNA for PEMT2, there is markedly decreased expression of CT in the hepatocytes (74). When PEMT activity is decreased in rapidly dividing cells, CT activity is elevated, providing further evidence for the coordination of the two pathways (60).

While it seems very likely that there is cross-talk between these two pathways, the mechanism for this is still unknown. As mentioned previously, there is evidence that cAMP

analogues increase PEMT activity (72, 75), presumably by a phosphorylation mechanism. There is also evidence that cAMP analogues can inhibit CT activity via phosphorylation by cAMP-dependent protein kinases (76). This relationship shows that the two pathways can be regulated in opposite directions by the same signal, presumably glucagon. This may be one mechanism to coordinate PC synthesis by the two pathways; however, this is probably not the only mechanism because it does not clearly explain the changes in PEMT and CT activity in rapidly dividing cells.

Function

Very low density lipoproteins

Very low density lipoproteins (VLDL) consist of apoB, triacylglycerol, cholesterol, cholesterol esters, and phospholipids and are made in the liver. Of the phospholipids, approximately 60% are PC and are located only on the surface (77). Full-sized VLDL particles are assembled on the endoplasmic reticulum, whereas nascent VLDL particles can be synthesized on the endoplasmic reticulum or the Golgi lumina (77). It has been shown that PC synthesized by PEMT is required for VLDL assembly (67).

Bile secretion

Lipids make up 23-29% of the total solids in bile, and approximately 80% of this is PC (78). Large quantities of PC are secreted into the bile every day. For example a 20 g mouse has ~20 mg of hepatic PC and secretes ~23 mg/d into the bile (59). Phosphatidylcholine and bile salts form a complex with cholesterol to solubilize the cholesterol. The concentrations of PC and bile salts determine how much cholesterol can be solubilized. Phosphatidylcholine alone can solubilize cholesterol, but at a much lower ratio than in combination with bile salts. Humans require multiple drug-resistant protein 3

(MDR3), a PC-specific flippase, in order to secrete PC into bile (59). Mice similarly require MDR2 for the secretion of PC into bile, and lack of this protein completely inhibits biliary PC secretion. When MDR2 knock-out mice (*Mdr2*$^{-/-}$) were bred with PEMT knock-out mice (*Pemt*$^{-/-}$) and fed a choline-deficient diet, they were able to adapt to a new low level of choline homeostasis and survive for at least 90 d (59). In comparison, *Pemt*$^{-/-}$ mice fed a choline-deficient diet who continued to secrete PC into bile died within 4-5 d. Furthermore, heterozygous MDR2 knock-out mice (*Mdr2*$^{+/-}$) secrete 40% less PC than do wild-type mice and have significantly less PEMT activity (79). This decreased activity is probably due to a decreased need for PC synthesis. These mice are less likely to develop non-alcoholic steatohepatitis (NASH) than wild-type mice fed a choline- and methionine-deficient diet, an animal model used to study NASH.

Cell signaling

Phosphatidylcholine accounts for ~50% of the membrane phospholipids (80). When a receptor in the plasma membrane is activated, it can subsequently activate a GTP-binding protein (G protein). Activation of a G protein is the first step in cell signaling and leads to activation of phospholipase C. Most forms of phospholipase C hydrolyze phosphatidylinositol bisphosphate to generate 1,2-*sn*-diacylglycerol, however, there are PC-specific phospholipase Cs (80). One of the products of PC hydrolysis is 1,3-*sn*-diacylglycerol, which maintains the signaling cascade generated by 1,2-*sn*-diacylglycerol. There are additional products from phosphatidylcholine hydrolysis that act as second messengers including phosphatidic acid, lysophosphatidylcholine, and free fatty acids such as arachidonic acid.

Besides acting as a second messenger, diacylglycerol can also be used to activate protein kinase C (PKC) by binding and causing a conformational change (80). This conformational change effectively "opens" up the catalytic site and activates the enzyme. PKC enhances substrates downstream to further amplify the signal that originally generated the diacylglycerol.

Choline deficiency leads to hepatocellular carcinomas in rats after 12 mo on a choline-deficient diet (81). There were alterations in 1,2-*sn*-diacylglycerol concentrations and PKC activity in these rats, which are important in intracellular signaling for cell growth, that may have led to the increase in carcinogenesis. There may also be an increased sensitivity to carcinogens due to increased DNA synthesis from the increased cell death and regeneration seen in choline deficiency. It was previously believed that choline deficiency caused increased apoptosis in hepatocytes due to an accumulation of lipids and decreased plasma membrane integrity, but evidence has shown apoptosis is induced before this occurs (80). It is now thought choline deficiency induces apoptosis through free radicals because inhibitors of free radical formation inhibit hepatocyte apoptosis during choline deficiency.

Factors that Affect Methyl Group Metabolism

Diabetes

General information

According to the American Diabetes Association (ADA), there are approximately 20.8 million people in the United States living with diabetes. The two major forms of diabetes are Type 1, or insulin-dependent, and Type 2, or non-insulin-dependent, diabetes mellitus. This review will focus primarily on Type 1 diabetes, as a Type 1 diabetic model was used to conduct the research presented later in this thesis.

Type 1 diabetes is an autoimmune disorder and is generally recognized as being a familial disorder. However, while genetics may play a strong role in the development of Type 1 diabetes, 90% of subjects with Type 1 diabetes do not have a first-degree relative with diabetes (82). During the development of Type 1 diabetes, β-cells, the insulin-producing cells of the pancreas, are destroyed with the preservation of α- and δ-cells of the pancreas. A viral infection may cause the β-cell destruction through cytotoxicity or triggering autoimmunity to β-cells by infecting the β-cells or lymphocytes. There are also other environmental factors that may influence the development of Type 1 diabetes, but these are not fully understood at this time.

There are many complications associated with Type 1 diabetes. Whereas insulin treatment does prevent death from diabetes, it does not completely prevent complications due to diabetes (83). One of the most common disorders associated with Type 1 diabetes is retinopathy. Retinopathy is damage to or lesions of the retina and is the leading cause of blindness for people between 20 and 74 years old in the United States (84). The development of retinopathy may be associated with the presence of neuropathy, or damage to the nerves as a result of high glucose concentrations or ischemia due to microvascular changes (85).

Nephropathy is another common complication of Type 1 diabetes. An increased urinary excretion of albumin marks the onset of nephropathy (85). Increased glomerular filtration rate (GFR) is associated with the early stages of nephropathy, with a rapid decline in GFR as end-stage renal failure progresses (85). Elevated blood lipids and homocysteine are highly correlated with renal failure.

Hypertension and heart disease are serious complications of diabetes. The incidence

of hypertension in diabetics is twice that of non-diabetics (86). There are four main types of hypertension in diabetes-- renal hypertension, hypertension without nephropathy, systolic hypertension, and orthostatic hypertension. Each of these types has a different pathogenesis and treatment. In Type 1 diabetic subjects, almost all who develop nephropathy also develop hypertension. Development of hypertension in a diabetic subject can have a major impact on their risk for coronary artery disease (CAD) (87).

As mentioned earlier, hyperhomocysteinemia is an independent risk factor for cardiovascular disease (4, 52). There is generally a decrease in plasma homocysteine levels in Type 1 diabetics with normal renal function. Plasma total homocysteine levels return to normal when insulin is given to Type 1 diabetic rats (46, 88). Unfortunately, renal failure is a common complication of diabetes and plasma homocysteine concentrations are highly correlated with kidney function; so, renal failure leads to hyperhomocysteinemia (89).

Fatty liver and NASH are additional complications of diabetes (90-93). NASH is characterized by inflammation and accumulation of fat and fibrous tissue in the liver. Although NASH is not always associated with diabetes, as much as 75% of the people with NASH have diabetes. In many people this is a stable condition, but in some cases NASH can lead to cirrhosis. The causes of NASH are unknown, but many people with NASH have elevated blood cholesterol and triacylglycerol concentrations. Currently there is no cure for NASH; so, it is important to find the causes of it so that it potentially can be prevented.

Effects on methyl group metabolism

A paper by Nieman *et al.* (6), offers an extensive view of the early changes that occur in methyl group metabolism in uncontrolled Type 1 diabetes using a streptozotocin-mediated diabetic rat model. They found an increase in GNMT activity and abundance and a decrease

in MS activity. The combined increase in GNMT activity and decrease in MS activity would suggest an increase in plasma homocysteine, but instead there was a decrease in plasma homocysteine. This decrease was probably the result of an increase in BHMT activity and an increase in CBS abundance, which re-methylates homocysteine to methionine and converts homocysteine to cysteine, respectively. There were increased concentrations of SAM and SAH, but no change in their ratio. Plasma homocysteine is initially decreased in diabetes but is elevated as renal failure progresses. The initial reduction in plasma homocysteine is probably the result of the characteristic increase in glomerular filtration rate with the onset of diabetes (94). The increase in GNMT, BHMT, and CBS activities, the decrease in MS activity, and the decrease in plasma homocysteine all seem to hold true in a Type 2 diabetic rat model (95). The activities of CBS, cystathionase, and MS, as well as plasma total homocysteine concentrations, all return to normal in Type 1 diabetic rats treated with insulin (46, 88).

The effect of diabetes on PEMT has not been consistent in past studies (61, 96-98). In the 1985 paper by Xue and Snoswell, they found PEMT activity to be reduced in alloxan-diabetic sheep (98). However, while they did find an increase in GNMT activity, they also found decreased activity of BHMT, no change in MS activity, and no change in CBS activity. These results seen in the sheep model seem to be very different than those seen in the rat model. The decrease in PEMT activity in alloxan-diabetic rats is supported in papers by Cabrero *et al.* and Hoffman *et al.* (96, 99). However, an increase in PEMT activity was seen in the cardiac subcellular membrane and brain of diabetic rats and mice, respectively (61, 97).

Retinoids

General information

Vitamin A is a term that describes a family of fat-soluble vitamins (100). The DRI (dietary reference intake) for vitamin A set by the Food and Nutrition Board of the Institute of Medicine is 700 μg/d and 770 μg/d for adult women and men, respectively, and a tolerable upper intake level of 3,000 μg/d for both genders. The vitamin A family can be further differentiated into provitamin A carotenoids and retinoids, which includes retinol, retinal, and retinoic acid. Some naturally occurring retinoids as well as synthesized retinoids have been shown to have pharmacological uses. One such retinoid, all-*trans*-retinoic acid, has both biological and pharmaceutical properties. All-*trans*-retinoic acid is marketed as Tretinoin® and Vesanoid® and is used in the treatment of acne and acute promyelocytic leukemia (APL) (101). Retinoic acid is thought to have its effects by influencing gene expression. Retinoic acid can bind retinoic acid receptor (RAR) and retinoid X receptor (RXR), which are nuclear transcription factors (**Figure 2.7**) (100). RAR and RXR then bind RAR and RXR response elements (RARE and RXRE) in the promoter regions of genes to influence gene expression.

The genes with RAR and RXR response elements often code for proteins that are regulators of cell development, proliferation, and differentiation. In the case of APL treatment, all-*trans*-retinoic acid induces the differentiation of leukemic promyelocytes into mature cells so that they can be eventually degraded instead of continuing to divide (102). Some common side effects associated with using pharmacological doses of retinoic acid include retinoic acid syndrome, elevated concentrations of triacylglycerols in blood (103, 104), depression/suicidal thoughts, and birth defects. Retinoic acid syndrome is characterized by fever, respiratory distress, hypotension, and renal failure (100). Retinoic

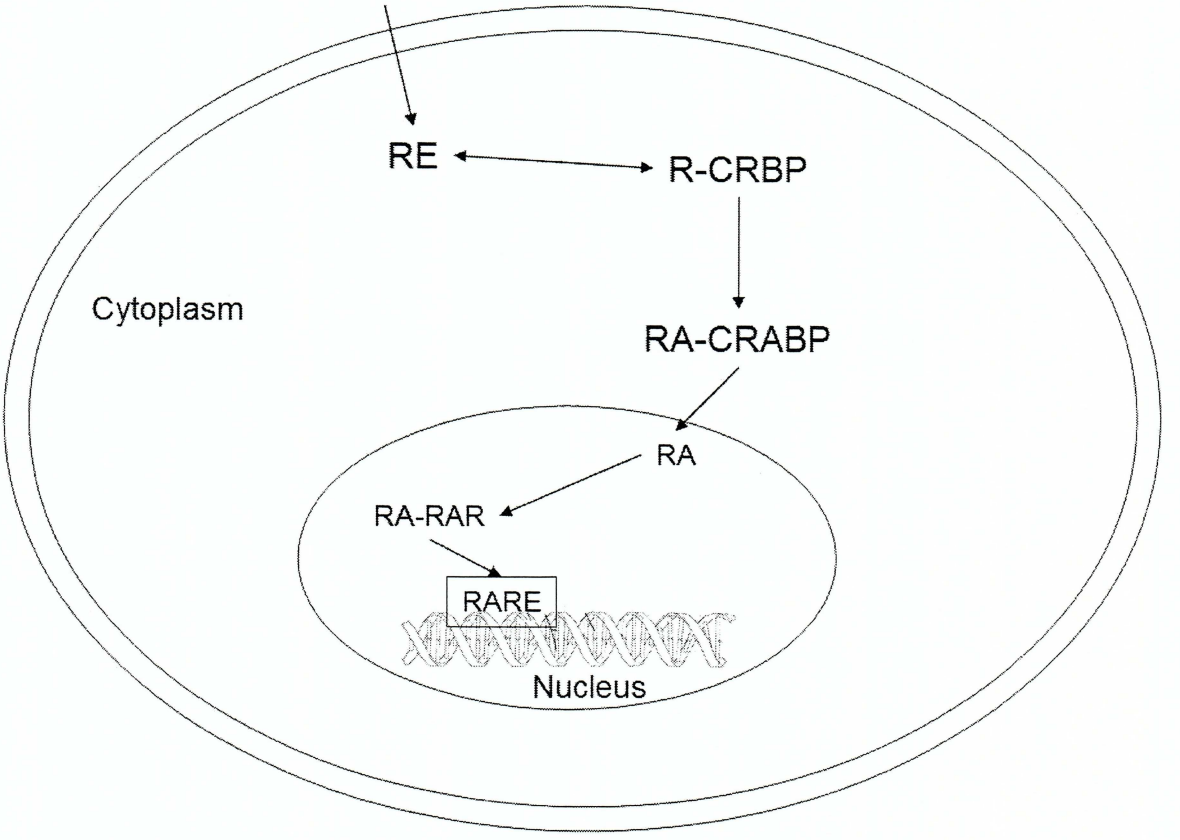


Figure 2.7. Schematic of how retinoic acid can influence gene expression
RE = Retinyl Ester; R = Retinol; CRBP = Cellular Retinol Binding Protein; RA = Retinoic Acid; CRABP = Cellular Retinoic Acid Binding Protein; RAR = Retinoic Acid Receptor; RARE = RAR Response Element

acid administration has also been associated with hepatic steatosis (103, 105, 106). Because of the severe side effects of retinoic acid administration, treatments can only be used for short periods of time and patients must be under constant medical supervision, including monthly check-ups when retinoic acid is used to treat acne.

Effects on methyl group metabolism

Aside from the other side effects of retinoic acid administrations, it has also been shown to disrupt methyl group metabolism (6, 107-113). Daily oral doses of retinoic acid

increased the activity, abundance, and mRNA of GNMT and lowered plasma homocysteine levels (6, 108-112). Additionally, MS activity was increased by administration of all-*trans*-retinoic acid (6). Fell and Steele showed that 1000 IU/g diet of retinol led to a decreased synthesis of PC by the PEMT pathway, which led to a decrease in hepatic PC levels as a percentage of total phospholipids (107). 13-*Cis*-retinoic acid, another form of retinoic acid, was shown to increase hepatic SAM levels, which led to a significant increase in the SAM to SAH ratio (6, 113). Conversely, in the study by Nieman *et al.* (6) there was no change in the SAM to SAH ratio when rats were dosed with all-*trans*-retinoic acid. Finally, yet another paper reported a significant decrease in hepatic SAM levels in rats fed 1000 IU/g of diet of retinol (6, 107). These findings suggest the effects of retinoids on methyl group metabolism may be linked to the form of vitamin A that is used in the study.

CHAPTER 3. STREPTOZOTOCIN-DIABETIC RATS HAVE INCREASED ACTIVITY OF HEPATIC PHOSPHATIDYLETHANOLAMINE N-METHYLTRANSFERASE[1]

A paper to be submitted to *Diabetes*

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Abstract

Diabetes and all-*trans*-retinoic acid (ATRA) have been shown to induce the expression of the folate-independent enzyme betaine-homocysteine *S*-methyltransferase (BHMT) and stimulate the activity of the folate-dependent enzyme methionine synthase, respectively, with a concurrent decrease in homocysteine concentrations. Phosphatidylcholine (PC) is an essential phospholipid that can be synthesized by two different pathways-- the CDP-choline pathway and the *S*-adenosylmethionine (SAM)-dependent methylation of phosphatidylethanolamine (PE) by phosphatidylethanolamine *N*-methyltransferase (PEMT). Recent studies have suggested that PEMT is a significant consumer of methyl groups from SAM and is major determinant of homocysteine pools. Thus, we investigated how diabetes and ATRA, both individually and together, affect SAM-dependent phospholipid methylation. Rats received a single injection of streptozotocin (60 mg/kg body weight) or vehicle followed by administration of ATRA (30 μmol/kg body weight) or vehicle for 5 d. The hepatic activity of PEMT was increased 50% in both diabetic rat groups, whereas administration of ATRA was without effect. The proportion of PC (% total phospholipids) was increased in all treatment groups, resulting in a decrease in the PE to PC ratio. The upregulation of phospholipid methylation in diabetic rats would be expected to increase homocysteine pools; however, total plasma homocysteine was decreased 30-35% in

all treatment groups as compared with the control group. Thus, under diabetic conditions alterations in the activity of PEMT was not directly correlated to changes in homocysteine concentrations. The increase in PEMT, as well as BHMT, taken together with the elevated biliary secretion of PC, may indicate an increased requirement for choline in the diabetic state.

Introduction

Phosphatidylcholine (PC)[5] is an important phospholipid component of cell membranes, hepatic lipoproteins, and bile and is often used in cell signaling pathways (114). Impaired PC biosynthesis can lead to an accumulation of triacylglycerols in the liver (115) and decreased secretion of VLDL (116), resulting in hepatosteatosis. PC can be synthesized by two different pathways. The cytidine 5'-diphosphate (CDP)-choline pathway involves three different enzymes: choline kinase, CTP:phosphocholine cytidylyltransferase (CT), and choline-phosphate transferase and is present in all nucleated cells (7, 117). 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 (9).

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 (42). It has been estimated that ~75% of the hepatic SAM pool is consumed by GAMT and ~15% by PEMT (42). However, in a recently published paper by Jacobs *et al.* (43), their findings suggest 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 (4, 52) and atherosclerosis (53). 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 (6, 108, 109, 112). The activity of glycine *N*-methyltransferase (GNMT), a key protein in the regulation of hepatic methyl group metabolism, was significantly increased following administration of streptozotocin (STZ) or ATRA, individually or in combination (6). The induction of GNMT would be expected to decrease the methyl group pool and increase homocysteine concentrations; however, homocysteine concentrations were decreased in diabetes and ATRA supplementation because of an increase in the activities of the folate-independent enzyme betaine homocysteine S-methyltransferase (BHMT) and the folate-dependent enzyme methionine synthase (MS), respectively. Because ATRA administration and diabetes disrupts 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 relationship between diabetes and PEMT have met with conflicting results (61, 96-99). Hoffman *et al.* (99) and Cabrero *et al.* (96) reported that hepatic PEMT activity was decreased in alloxan-diabetic rats, which is similar to the decrease found in alloxan-diabetic sheep (98). For extrahepatic tissues, the activity of PEMT was increased in the cardiac subcellular membrane and brain of STZ-diabetic rats and mice, respectively (61, 97). Although PEMT activity was not determined, Wijekoon *et al.* (95) 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. For example, glucagon has been shown to increase PEMT activity in cultured hepatocytes (72, 75, 118). Therefore, it is important to determine how diabetes, alone and in combination with ATRA administration, 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, PerkinElmer Life and Analytical Sciences (Boston, MA); STZ, ATRA, and SAM, Sigma-Aldrich (St. Louis, MO); and soy PE and soy PC standards, Avanti Polar Lipids, Inc. 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 both studies, male Sprague-Dawley (Harlan Sprague-Dawley, Indianapolis, IN) rats (125-149 g) were housed in plastic cages with a 12-hour light:dark cycle and given food and water *ad libitum*. The control diet has been previously described (113). For experimental study 1, 25 rats were randomly assigned to receive a single intraperitoneal injection of STZ (60 mg/kg body weight) on d 0 and sacrificed on d 3, 5, or 7. Non-diabetic control rats were injected with the vehicle (10 mmol/L citrate buffer, pH 4.5) and sacrificed on d 0 and 7. For experimental study 2, 24 rats were randomly assigned to one of four treatment groups: control, STZ, ATRA, or both

treatments (STZ+ATRA). After diabetes initiation, ATRA was orally administered daily in corn oil at a dose of 30 μmol/kg body weight for 5 d. For both studies, rats were anesthetized with an intraperitoneal injection of ketamine:xylazine (90:10 mg/kg body weight) and blood samples were collected via cardiac puncture in heparinized syringes. Samples of whole blood were used to determine blood glucose using a commercial kit (Sigma-Aldrich, St. Louis, MO). Additional whole blood samples were centrifuged at 4,000 × g for 5 min and the plasma fraction was stored at -70°C until analysis. Livers were rapidly removed and a one-gram portion was used for preparation of microsomes; remaining liver samples were snap-frozen in liquid nitrogen and stored at -70°C.

Isolation of liver microsomes

Approximately 1 g of liver was homogenized with a Polytron-Aggregate apparatus (Brinkmann Instruments Inc., Westbury, NY) 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 16,000 × g for 20 min at 4°C followed by centrifugation of the supernatant (1-2 mL) at 105,000 × g for 60 min at 4°C. The resulting supernatant was discarded and the pellet was resuspended in 400 μL of 0.25 mol/L sucrose. The microsomal fraction was stored at -70°C until determination of PEMT activity. The protein concentration of the microsomal fraction was determined by the Bradford method (119) using a commercial kit (Coomassie Plus, Pierce).

PEMT activity

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.* (120) with modifications. In brief, the reaction mixture contained 0.1 mmol/L SAM, 2 μCi *S*-adenosyl-L-[*methyl*-^{3}H]methionine, 10 mmol/L HEPES, 4

mmol/L dithiothreitol (DTT), 5 mmol/L $MgCl_2$, and 750 μg protein in a final volume of 550 μL. Exogenous PE was not added to the reaction mixture because it has not been shown to significantly increase the reaction rate (121, 122). Following incubation in a 37°C water bath for 10 min, the reaction was terminated by pipetting 100 μL of the assay mixture into 2 mL chloroform:methanol:2 N HCl (6:3:1, v:v:v) in duplicate. The chloroform phase was washed three times with 1 mL 0.5 mol/L KCl in 50% methanol, transferred to a glass scintillation vial, and dried at room temperature. The dried lipid fraction was reconstituted in 5 mL Scintverse® (Fisher Scientific, Pittsburgh, PA) and the radioactivity was determined by liquid scintillation counting.

Plasma homocysteine

Total homocysteine (free and protein-bound) concentrations were determined according to a previously described method (123). Briefly, 300 μL of plasma, 30 μL of 1 mmol/L *N*-acetylcysteine, and 30 μL of 10% tributylphosphate in dimethylformamide were incubated at 4°C for 30 min. To terminate the reaction, 300 μL of 10% trichloroacetic acid and 1 mmol/L EDTA were added and samples were centrifuged at 1,000 × g for 5 min. For derivatization, a 100 μL sample of the supernatant was transferred to a tube containing 20 μL of 1.55 mol/L sodium hydroxide, 250 μL of borate buffer (0.125 mol/L, pH 9.5), and 100 μL of 4-fluoro-7-sulfobenzofurazan (1 g/L). Samples were incubated for 60 min at 60°C, filtered, and analyzed using HPLC with fluorometric detection.

Lipid analysis

Frozen liver samples were thawed and total liver lipids were extracted by the method of Folch *et al.* (124). A fraction of the Folch extract was dried and weighed to quantify total liver lipids. To determine specific phospholipid concentrations, total lipid extracts were

fractionated on a silica Sep-Pak cartridge (Waters, Milford, MA). 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 μL chloroform, and a 20 μL aliquot was injected onto a HPLC system for quantification of phosphatidylethanolamine, phosphatidylcholine, and total phospholipids using a Pholipidec Soy Lecithin Column (Astec, Whippany, NJ) by the method of Palacios and Wang with modifications (125). Soy PE and PC standards (Avanti Polar Lipids, Alabaster, AL) were used to quantify hepatic concentrations of PE and PC. Using plasma from whole blood samples and Folch lipid extracts of liver, triacylglycerols were analyzed using a commercial kit (Sigma-Aldrich, St. Louis, MO).

Statistical analysis

The mean values from each treatment group were compared using one-way ANOVA at a significance level of 5%. A one-way ANOVA on ranks was performed when the test for normality failed. If there was a significant difference among the means, comparisons were accomplished 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, Chicago, IL).

Results

Hepatic PEMT activity was rapidly increased by diabetes. Because insulin release after STZ injection may vary considerably, it was first important to determine the hepatic activity of PEMT as a function of time from the onset of diabetes. The activity of

PEMT increased over 7 d following the injection of STZ with the activity being significantly elevated 58% and 72% on d 5 and 7, respectively, as compared to control values (**Figure 3.1**).

ATRA administration was without affect on hepatic PEMT activity. The second study also demonstrated that diabetes increased PEMT activity, with or without ATRA administration. Both groups of diabetic rats (STZ and STZ+ATRA) exhibited significantly higher PEMT activity after 5 d as compared to control values (**Figure 3.2**); however, administration of ATRA alone or in combination with STZ had no effect on PEMT activity.

Plasma homocysteine concentrations were reduced by both diabetes and ATRA. Plasma homocysteine levels were reduced 32% in the STZ group (11.3 ± 0.8 μmol/L) as compared with the control group (16.7 ± 1.6 μmol/L) (**Figure 3.3**). Similarly, plasma homocysteine concentrations were reduced ~35% in the ATRA (10.9 ± 01.0 μmol/L) and the STZ+ATRA group (10.1 ± 1.1 μmol/L). Therefore, no correlation was demonstrated between the activity of PEMT and circulating homocysteine concentrations ($r = 0.109$, $P = 0.611$).

Diabetes and ATRA administration increased the hepatic PE: PC ratio. There were no significant differences in total phospholipids among the four treatment groups (data not shown). Total hepatic PE concentrations and the percent PE of total phospholipids were reduced in the STZ and ATRA groups as compared to control values (**Figure 3.4**). The 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 to control values.

Triacylglycerols accumulated in the liver with ATRA administration, which was modulated by the diabetic state. Liver TAG concentrations were increased ~150% in the ATRA group which consequently had higher liver lipid concentrations (**Table 3.1**). Whereas STZ alone had no effect on TAG concentrations, STZ in combination with ATRA resulted in a 400% increase in plasma TAG concentrations, with no difference in liver TAG concentrations as compared to the control group. These data suggest there may be an increased ability to secrete TAG from the liver in an acute diabetic state.

Discussion

Regulation of PEMT for the SAM-dependent synthesis of phosphatidylcholine is an important transmethylation reaction and represents a significant use of methyl groups. PC synthesis is required for cell membranes, bile secretion, and the ability to secrete VLDL from the liver. We have shown in these studies that the activity of PEMT is rapidly elevated in a type 1 diabetes model, resulting in a decrease in the PE:PC ratio. Taken together with our previous finding that BHMT activity is elevated under diabetic conditions (6), 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 re-methylation. It is not clear whether the increased production of PC is compensatory for the loss of choline for homocysteine re-methylation 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 (126). There is evidence that PEMT activity can be regulated by the need for PC synthesis. A study by Igolnikov and Green (79) showed decreased PEMT activity in *Mdr*$^{+/-}$ mice as compared with wild-type mice. The *Mdr*$^{+/-}$ mice secrete ~40% biliary PC than the wild-type mice and therefore have a

decreased need for PC synthesis.

Liver triacylglycerolss accumulated with ATRA administration, similar to the results seen by Schalinske and Steele (106) with 13-*cis*-retinoic acid supplementation. However, in the diabetic group that received ATRA supplementation, plasma triacyglycerols were elevated, but not liver triacylglycerols. These data suggest there is an increased secretion of VLDL in the diabetic state and this is what prevented liver TAG accumulation due to ATRA. PC is the predominant phospholipid in VLDL, so the increased VLDL secretion would require increased PC synthesis.

In addition to phospholipid metabolism, these results have significant implications for the relationship between PEMT function and homocysteine homeostasis. Recent studies have shown that regulation of PEMT is tightly correlated to homocysteine production and bile synthesis. Using *Pemt*$^{-/-}$ knock out mice, it has been demonstrated that a lack of PEMT expression resulted in low circulating concentrations of homocysteine, whereas homocysteine secretion was enhanced following induction of PEMT expression in hepatoma cells (68, 127). Likewise, CTP:phosphocholine cytidylyltransferase-α knockout mice exhibited a 2-fold increase in PEMT activity, similar to our findings in diabetic rats, that resulted in an increase in homocysteine production and secretion (43). A recent paper further demonstrates that 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 (59). Taken together with our findings, diabetes appears to be another factor that has a major impact on the regulation of PEMT and PC synthesis. This is supported by the observation that biliary PC secretion is elevated in diabetic rats (126).

Of particular interest in our studies was the finding that, although PEMT activity was

elevated in diabetic rats, it did not correlate with changes in homocysteine concentrations in the plasma. All diabetic rats exhibited an elevation in hepatic PEMT activity and reduced homocysteine levels, presumably owing to 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 re-methylation. 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 diabetic rats (88, 128).

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. It has been shown that incubating hepatocytes with glucagon or cAMP increases PEMT activity, potentially the result of altering its phosphorylation state (75). Other factors that affect PEMT activity include B_6 status (35) and the ratio of SAM/ SAH (129), although we did not find that methylation capacity was altered in diabetic rats (6). Because many of the findings reported for type 1 diabetes models have also been demonstrated in a type 2 model using ZDF rats (95), alterations in substrate or cofactor availability may be a more plausible explanation than the direct effects of hormonal imbalance.

Our previous studies using diabetic and ATRA-treated rats demonstrated a clear interaction between these two treatments with respect to folate-dependent and folate-independent re-methylation of homocysteine, as well as its catabolism by the transsulfuration pathway (6). Moreover, the combination of diabetes or glucocorticoid treatment with ATRA

administration exerted an additive effect on inducing GNMT expression (6, 112). 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 (107, 130); 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 upregulation of SAM-dependent phospholipid methylation and is characterized by a state of hypohomocysteinemia, due to elevations in folate-independent re-methylation of homocysteine. Choline is important for PC synthesis and is significant source of methyl groups in the liver through its oxidation to betaine. For ZDF rats, Wijekoon *et al.* (95) 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, Igolnikov and Green (79) found increased PEMT activity in mice fed a choline deficient diet. 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. This latter implication is important with respect to the progression of diabetes, as it has been well documented that both type 1 and type 2 diabetes eventually progresses to a state of hyperhomocysteinemia, particularly when diabetic

nephropathy is evident (95, 131-136). The temporal and tissue-specific regulation of PEMT and homocysteine will be an important area for future research, in large part owing to the association of hyperhomocysteinemia with vascular disease and other pathological conditions. Likewise, the potential benefit of choline and/or betaine supplementation as a viable diabetes intervention strategy needs to be examined.

Acknowledgements

The authors wish to thank Liping Wang and Dr. Tong Wang for the phospholipid analysis.

Footnotes

[1]This work was supported in part by the American Diabetes Association and the Cancer Research and Prevention Foundation

[2]Graduate student and Associate Professor, respectively, Department of Food Science and Human Nutrition, Iowa State University

[3]Primary researcher and author

[4]Author for correspondence

[5]The abbreviations used are: ATRA, all-*trans*-retinoic acid; BHMT, betaine homocysteine *S*-methyltransferase; CDP, cytidine 5'-diphosphate; CT, CTP:phosphocholine cytidylyltransferase; GAMT, guanidinoacetate *N*-methyltransferase; GNMT, glycine *N*-methyltransferase; MS, methionine synthase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; STZ, streptozotocin; TAG, triacylglycerol; and, VLDL, very low density lipoprotein.

Figure Legends

FIGURE 3.1. Streptozotocin (STZ)-mediated diabetes rapidly increased the activity of hepatic phosphatidylethanolamine *N*-methyltransferase (PEMT). Rats received an intraperitoneal injection of either STZ (60 mg/kg body weight) or vehicle (10 mmol/L citrate buffer, pH 4.5) on d 0 and were sacrificed on d 0, 3, 5, or 7. Liver samples were collected and PEMT activity was determined as described under "Materials and Methods". Data are expressed as mean ± SEM (n=6). Values with an *asterisk* indicates that they were significantly different from control (d 0) values, $P < 0.05$.

FIGURE 3.2. Streptozotocin (STZ)-mediated diabetes increased phosphatidylethanolamine *N*-methyltransferase (PEMT) activity, whereas all-*trans*-retinoic acid (ATRA) administration was without effect. Rats were injected with STZ (60 mg/kg body weight) or vehicle (10 mmol/L citrate buffer) on d 0 followed by administration of ATRA (30 μmol/kg body weight) or vehicle (corn oil) for 5 d. Liver samples were collected and PEMT activity was determined as described under "Materials and Methods". Data are expressed as mean ± SEM (n=6). Bars denoted with an *asterisk* indicates that they were significantly different from control values, $P < 0.05$.

FIGURE 3.3. Streptozotocin (STZ)-mediated diabetes and all-*trans*-retinoic acid (ATRA) administration decreased plasma total homocysteine concentrations. Rats were injected with STZ (60 mg/kg body weight) or vehicle (10 mmol/L citrate buffer) on d 0 followed by administration of ATRA (30 μmol/kg body weight) or vehicle (corn oil) for 5 d. Blood samples were collected via cardiac puncture, centrifuged, and the plasma fraction used

for the determination of homocysteine concentrations by HPLC as described under "Materials and Methods". Data are expressed as mean ± SEM (n=6). Bars denoted with an *asterisk* indicates that they were significantly different from control values, $P < 0.05$.

FIGURE 3.4. Streptozotocin (STZ)-mediated diabetes and all-*trans*-retinoic acid (ATRA) administration decreased phosphatidylethanolamine (PE) and increased phosphatidylcholine (PC) concentrations. Rats were injected with STZ (60 mg/kg body weight) or vehicle (10 mmol/L citrate buffer) on d 0 followed by administration of ATRA (30 μmol/kg body weight) or vehicle (corn oil) for 5 d. Liver samples were collected and phospholipid concentrations were determined as described under "Materials and Methods". Data are expressed as mean ± SEM (n=6). Values denoted with *different letters* indicates that they were significantly different from control values, $P < 0.05$. The statistical results were the same for all three values (PE, PC, PE:PC) within each treatment group.

Tables and Figures

Table 3.1

Triacylglycerol compositions of rat livers and plasma from the control, diabetic (STZ, 60 mg/kg body weight), all-*trans*-retinoic acid supplemented (ATRA, 30 µmol/kg body weight), and STZ+ATRA treatment groups

Values are expressed as mean ± SEM (n=6). Values within a row with a different letter subscript are significantly different, $P < 0.05$.

	Control	STZ	ATRA	STZ+ATRA
Triacylglycerols				
Liver *(mg/g liver)*	3.48 ± 0.51^{a}	1.92 ± 0.18^{a}	8.60 ± 1.10^{b}	2.00 ± 0.12^{a}
Plasma *(mmol/L)*	0.97 ± 0.15^{a}	1.91 ± 0.66^{a}	1.45 ± 0.20^{a}	4.78 ± 1.03^{b}

Figure 3.1

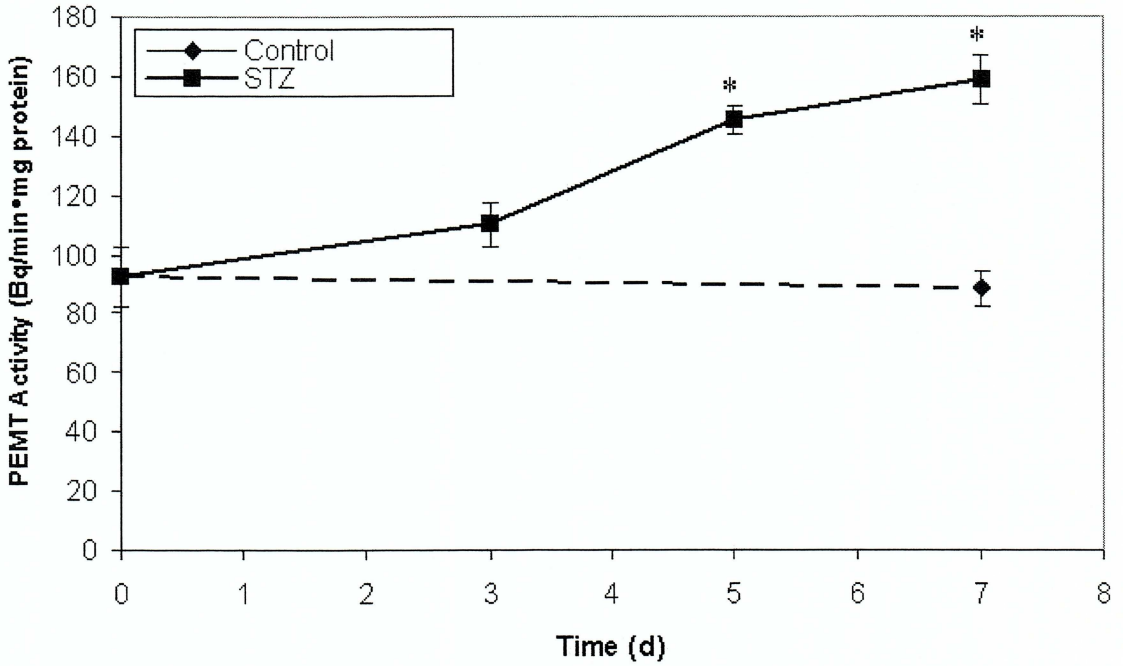

Control
STZ
PEMT Activity (Bq/min•mg protein)
Time (d)
0
20
40
60
80
100
120
140
160
180
0
1
2
3
4
5
6
7
8
*
*

Figure 3.2

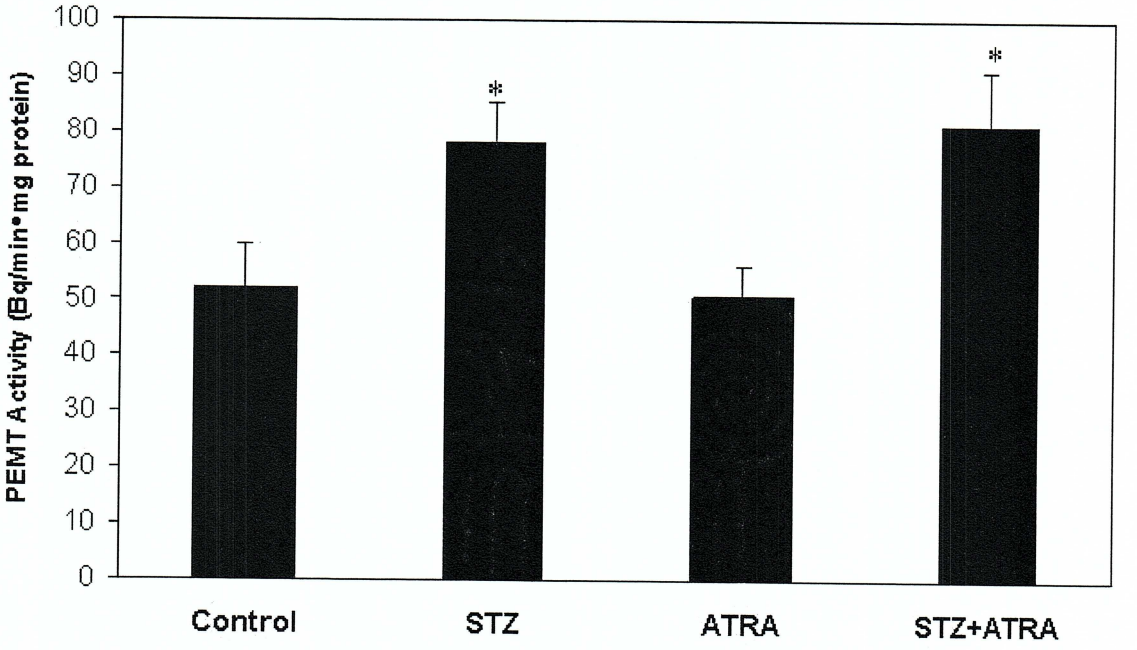

PEMT Activity (Bq/min•mg protein)
100
90
80
70
60
50
40
30
20
10
0
*
*
Control
STZ
ATRA
STZ+ATRA

Figure 3.3

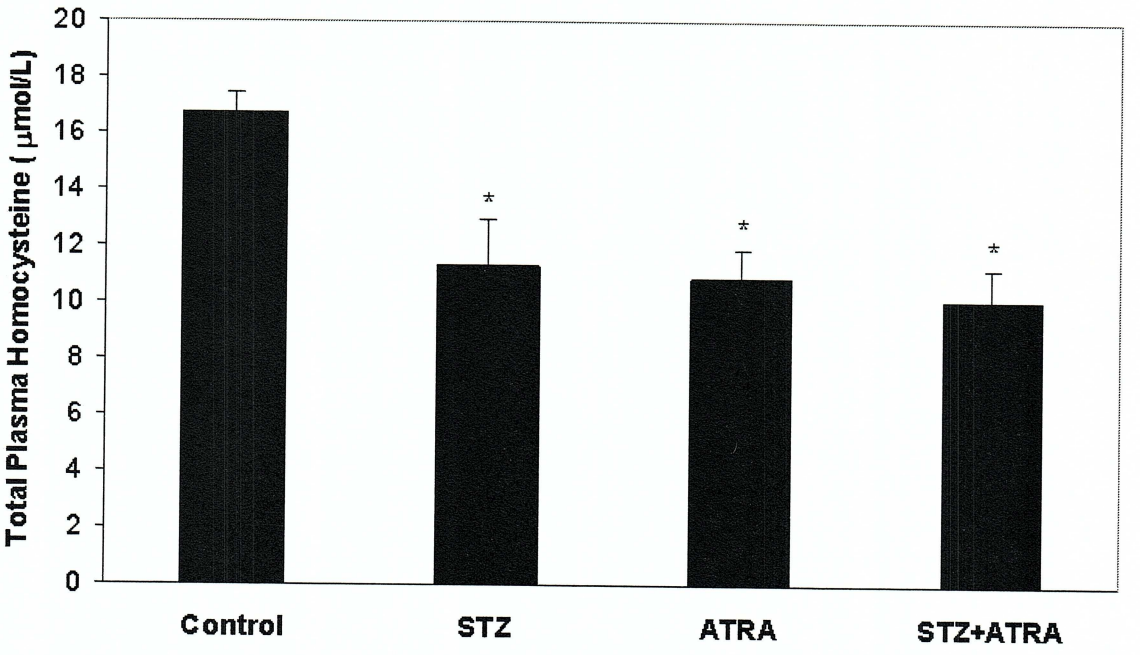
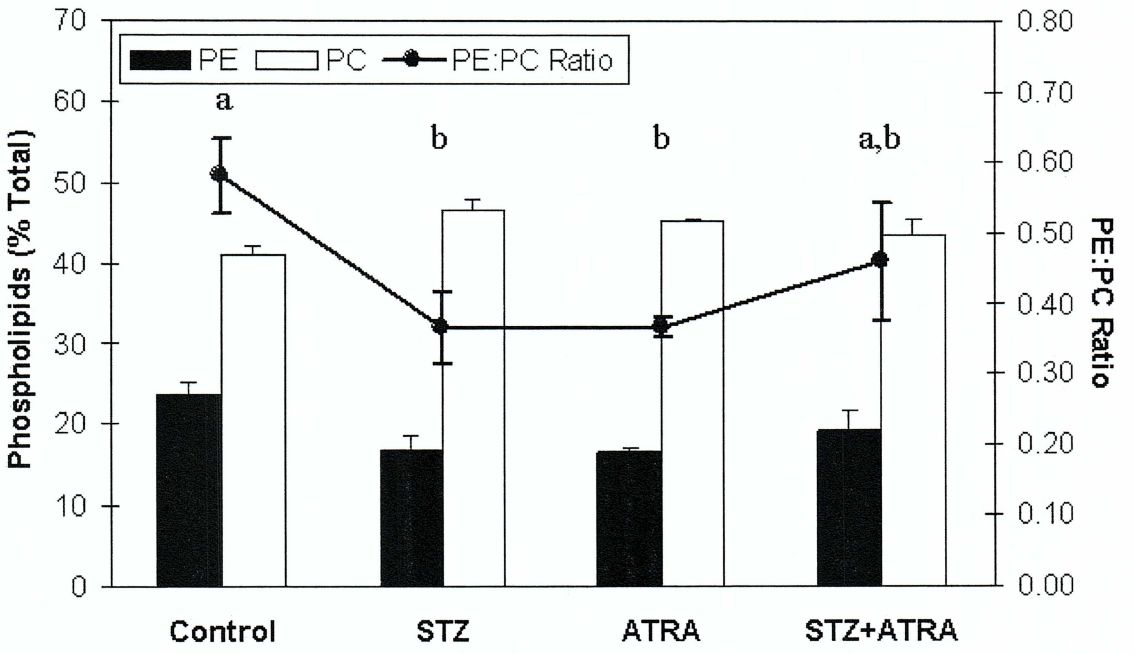

Total Plasma Homocysteine (µmol/L)
20
18
16
14
12
10
8
6
4
2
0
*
*
*
Control
STZ
ATRA
STZ+ATRA

Figure 3.4


PE
PC
PE:PC Ratio
Phospholipids (% Total)
PE:PC Ratio
70
60
50
40
30
20
10
0
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
a
b
b
a,b
Control
STZ
ATRA
STZ+ATRA

CHAPTER 4. CHOLINE AND BETAINE SUPPLEMENTATION DID NOT ATTENUATE EFFECTS OF DIABETES ON PHOSPHATIDYLETHANOLAMINE N-METHYLTRANSFERASE[1]

Abstract

Phosphatidylcholine (PC) is an essential compound in cell membranes, VLDL, cell signaling, and bile secretion. PC synthesis in the liver can be achieved by two different pathways, the CDP-choline pathway and the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway. The CDP-choline pathway synthesizes PC directly from choline, whereas PC synthesis by the PEMT pathway is achieved via methylation of phosphatidylethanolamine (PE) and is not directly dependent on choline. Betaine-homocysteine *S*-methyltransferase (BHMT) utilizes betaine to re-methylate homocysteine to methionine. Betaine can be obtained directly from the diet or through oxidation of choline. Previously, we have shown PEMT and BHMT activities are elevated in the diabetic state. This study was conducted to determine if choline or betaine supplementation would decrease PEMT activity as a result of an increased availability of choline for the CDP-choline pathway. Twenty-four male Sprague-Dawley rats were randomly assigned to be fed the control diet or to be supplemented with 1% choline or betaine. Following a 10-d period on their respective diets, half the rats on the control diet and all of the rats in the supplemented groups received a single injection of streptozotocin (STZ, 60 mg/kg body weight). PEMT activity was elevated 107-127% in all STZ treatment groups. Supplementation had no effect on PEMT activity, whereas GNMT activity and abundance were increased 147% and 234%, respectively. CBS protein abundance was elevated in all STZ groups regardless of diet. These results suggest PEMT activity is not elevated to account for decreased choline

availability to the CDP-choline pathway. It is more likely that PEMT activity is hormonally regulated in the diabetic state.

Introduction

Choline and betaine are found in a wide variety of food sources. Choline can be metabolized to phosphatidylcholine, sphingomeylin, acetylcholine, platelet-activating factor, and betaine (16). Phosphatidylcholine (PC)[2] is an important phospholipid for cell membranes, cell signaling, VLDL, and bile secretion (114). PC can be synthesized by two different pathways in the liver. The first of these pathways, which produces about 70% of the hepatic PC, is the CDP-choline pathway. Choline is metabolized to PC in a series of reactions with the rate limiting step being the conversion of phosphocholine to cytidine diphosphocholine (CDP-choline) by the enzyme CTP:phosphocholine cytidylyltransferase (CT) (16). This conversion is also the regulated step of PC synthesis by the CDP-choline pathway. The other pathway for hepatic synthesis of PC is the methylation of phosphatidylethanolamine (PE) to PC by phosphatidylethanolamine *N*-methyltransferase (PEMT) (16). PEMT is not necessary for survival as *Pemt*$^{-/-}$ knock-out mice on a choline sufficient diet survive, but develop hepatic steatosis (137). However, hepatocytes lacking PEMT secreted 50% less triacylglycerols in apoB-100-containing VLDL than hepatocytes with PEMT, while there was no change in the secretion of PC (127). There was no change in apoB-100 secretion in mice fed a choline-deficient diet for 3 or 21 d (138). This evidence suggests PC synthesis via PEMT is necessary for normal VLDL secretion and is a possible reason why *Pemt*$^{-/-}$ knock-out mice develop hepatic steatosis regardless of the dietary choline intake.

Previously, we have shown the streptozotocin (STZ)-mediated diabetes in rats

increases the activity of hepatic PEMT (see previous chapter). This is significant because PEMT is a major consumer of methyl groups in the liver and so has the ability to considerably contribute to homocysteine concentrations. Plasma total homocysteine goes down with the onset of Type 1 diabetes, but becomes elevated as nephropathy due to diabetes progresses (94). Homocysteine concentrations go down with diabetes due to the increased re-methylation of homocysteine to methionine by betaine-homocysteine *S*-methyltransferase (BHMT) and the increased transsulfuration of homocysteine to cysteine by cystathionine β-synthase (CBS) (6). The increased activity of BHMT leads to an increased usage of betaine in the liver. This may cause a decrease in hepatic betaine concentrations, which has been shown in a Type 2 diabetic model (95). Choline has the ability to be oxidized to betaine and in fact as much as 60% of choline may be oxidized to betaine in the liver (11). The ability of choline to be oxidized raises the possibility that, not only are hepatic betaine concentrations diminished in diabetes, but also choline. With less available choline, PC synthesis via the CDP-choline pathway would be diminished, making it necessary for the PEMT pathway to produce more choline. There has also been evidence that there is increased biliary PC secretion in the diabetic state, increasing the need for PC synthesis (126). We investigated the effect of choline and betaine supplementation on PEMT activity to determine if in fact there is an increased choline or betaine requirement in the diabetic state.

Materials and Methods

Chemicals

Reagents were obtained from the following: *S*-adenosyl-L-[*methyl*-^{3}H]methionine, PerkinElmer Life and Analytical Sciences (Boston, MA); STZ and SAM, Sigma-Aldrich (St. Louis, MO); betaine anhydrous (98% pure), Acros Organics (Belgium). 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. Twenty-four male Sprague-Dawley (Harlan Sprague-Dawley, Indianapolis, IN) rats (125-149 g) were housed in plastic cages with a twelve-hour light:dark cycle and given food and water *ad libitum*. All rats were randomly assigned to receive one of three diets. Twelve rats were fed the control diet, containing 0.2% choline bitartrate, which has been previously described (113), six rats were fed the control diet supplemented with choline to the level of 1%, and six rats were fed the control diet plus 1% betaine anhydrous. This level of supplementation was chosen because a similar level was used in other rodent studies (139, 140). After the rats had been on their respective diets for 10 days, 6 of the rats on the control diet and all of the rats on the supplemented diets were injected with streptozotocin (STZ; 60 mg/kg body weight). The remaining six rats on the control diet were injected with the vehicle (10 mmol/L citrate buffer, pH 4.5). On d 15, rats were anesthetized with an intraperitoneal injection of ketamine:xylazine (90:10 mg/kg body weight) and blood samples were collected via cardiac puncture in heparinized syringes. Samples of whole blood were used to determine blood glucose using a commercial kit (Sigma-Aldrich, St. Louis, MO). A cut-off of 20 mmol/L blood glucose was used to determine if the rats were diabetic or not. There were two rats in the streptozotocin group on control diet and one rat in the streptozotocin group on the 1% choline diet that did not reach this cut-off and were excluded from additional analyses. Additional whole blood samples were centrifuged at 4,000 × g for 5 min and the plasma fraction was stored at -70°C until analysis. Livers were rapidly removed and a one-gram portion was used for preparation of

microsomes; remaining liver samples were snap-frozen in liquid nitrogen and stored at -70°C.

Isolation of liver microsomes

Approximately 1 g of liver was homogenized with a Polytron-Aggregate apparatus 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 16,000 × g for 20 min at 4°C followed by centrifugation of the supernatant (1-2 mL) at 105,000 × g for 60 min at 4°C. The resulting supernatant was discarded and the pellet was resuspended in 400 µL of 0.25 mol/L sucrose. The microsomal fraction was stored at -70°C until determination of PEMT activity. For GNMT activity and GNMT and CBS abundance, 1 mL of the supernatant from the 16,000 x g centrifugation was added to 10 µL of 2-mercaptoethanol and stored at -70°C until the time of analysis. Protein concentrations of the microsomal fraction and supernatant were determined by the Bradford method (119) using a commercial kit (Coomassie Plus, Pierce).

PEMT activity

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.* (120) with modifications. In brief, the reaction mixture contained 0.1 mmol/L SAM, 2 µCi *S*-adenosyl-L-[*methyl*-^{3}H]methionine, 10 mmol/L HEPES, 4 mmol/L dithiothreitol (DTT), 5 mmol/L $MgCl_2$, and 750 µg protein in a final volume of 550 µL. Following incubation in a 37°C water bath for 10 min, the reaction was terminated by pipetting 100 µL of the assay mixture into 2 mL of chloroform:methanol:2 N HCl (6:3:1, v:v:v) in duplicate. The chloroform phase was washed three times with 1 mL of 0.5 mol/L KCl in 50% methanol, transferred to a glass scintillation vial, and dried at room temperature.

The dried lipid fraction was reconstituted in 5 mL Scintverse® (Fisher Scientific, Pittsburgh, PA) and the radioactivity was determined by liquid scintillation counting.

GNMT activity

The enzymatic activity of GNMT was measured based on the method of Cook and Wagner (141) with slight modifications (112). The assay was performed in triplicate using 250 μg protein and a reaction mixture containing the following: 200 mM Tris buffer (pH 9.0), 2 mM glycine, 5 mM dithiothreitol, and 0.2 mM *S*-adenosyl-L-[*methyl*-^{3}H]methionine. Following a 30 min incubation period at 25°C, 10% trichloroacetic acid was added to stop the reaction and activated charcoal was used to remove the unreacted SAM. Samples were centrifuged at 14,000 × *g* and a 200 μL aliquot of the supernatant was added to 5 mL Scintverse® (Fisher Scientific, Pittsburgh, PA). Radioactivity was determined by liquid scintillation counting.

GNMT and CBS protein abundance

Three samples from each treatment group were randomly chosen to determine abundance of GNMT and CBS proteins using immunoblotting methods described previously (112). In brief, a 10-20% gradient SDS-polyacrylamide gel was utilized to separate the 32-kDa monomer subunit of GNMT and the 64-kDa subunit of CBS. Proteins were transferred to nitrocellulose paper and incubated with a 1: 2,500 dilution of the monoclonal GNMT antibody (142) overnight at 4°C. The membrane was then incubated with a 1: 5,000 dilution of a goat anti-mouse horseradish peroxidase secondary antibody for 1 hr at room temperature. GNMT protein abundance was detected using chemiluminescence and exposure of the membrane to Kodak X-Omat AR film. After detecting GNMT abundance, the membrane was stripped using Restore™ Western Blot Stripping Buffer (Pierce). The

membrane was incubated with a 1: 80,000 dilution of the polyclonal CBS antibody overnight at 4°C. Following incubation with the primary antibody, the membrane was incubated with a 1: 5,000 dilution of a goat anti-rabbit horseradish peroxidase secondary antibody for 1 hr at room temperature. CBS protein abundance was detected in the same manner as GNMT protein abundance. Band density was determined using SigmaGel software (SPSS, Chicago, IL).

Plasma homocysteine

Total homocysteine (free and protein-bound) concentrations were determined according to a previously described method (123). Briefly, 300 μL of plasma, 30 μL 1 mmol/L *N*-acetylcysteine, and 30 μL 10% tributylphosphate in dimethylformamide were incubated at 4°C for 30 min. To terminate the reaction, 300 μL of 10% trichloroacetic acid and 1 mmol/L EDTA were added and samples were centrifuged at 1,000 × g for 5 min. For derivatization, a 100 μL sample of the supernatant was transferred to a tube containing 20 μL 1.55 mol/L sodium hydroxide, 250 μL borate buffer (0.125 mol/L, pH 9.5), and 100 μL 4-fluoro-7-sulfobenzofurazan (1 g/L). Samples were incubated for 60 min at 60°C, filtered, and analyzed using HPLC with fluorometric detection.

Lipid analysis

Frozen liver samples were thawed and total liver lipids were extracted by the method of Folch *et al.* (124). A portion of the extract was dried and weighed to determine total liver lipids. Using plasma from whole blood and Folch lipid extracts of liver, triacylglycerols were determined by a spectrophotometer and a commercial kit (Sigma-Aldrich, St. Louis, MO).

Statistical analysis

The mean values from each treatment group were compared using one-way ANOVA at a significance level of 5%. If there was a significant difference among the means, comparisons were accomplished 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, Chicago, IL).

Results

PEMT Activity was not attenuated by choline or betaine supplementation. PEMT activity was elevated by 107-127% in the STZ groups as compared to the control (**Figure 4.1**). There was no effect of choline or betaine supplementation on PEMT activity in the STZ-diabetic rats.

GNMT activity and abundance were significantly increased with betaine supplementation. GNMT activity was increased by 58% and 72% in the STZ groups on the control diet and 1% choline diet, respectively (**Figure 4.2**). 1% betaine supplementation in the STZ-diabetic rats caused an even greater increase in GNMT activity of 147%. The changes in GNMT activity were reflected in an increased protein abundance of GNMT in the STZ-diabetic rats supplemented with 1% choline or 1% betaine.

CBS protein abundance was not altered by choline or betaine supplementation. CBS protein abundance was increased to 158-193% of the control in all STZ-treatment groups (**Figure 4.3**). However, there was no difference in CBS protein abundance among the different dietary groups.

Plasma total homocysteine was not significantly different among the treatment groups. Due to a wide variability of homocysteine concentrations within each treatment

group, there was no significant difference in plasma total homocysteine between the groups (**Figure 4.4**). There did appear to be a trend towards increased homocysteine with increased supplementation among the diabetic groups.

Liver lipids were significantly reduced in the diabetic treatment groups. There was a decrease in total liver lipids of 14-21% in all STZ-diabetic groups as compared to the control group (**Table 4.1**). There were no significant differences in plasma triglycerides due to large variations within treatment groups and small sample sizes. However, there did appear to be a trend towards increased plasma TAG in the STZ-diabetic groups.

Discussion

As a major consumer of hepatic methyl groups, PEMT can have a significant effect on homocysteine concentrations. It is important to keep plasma homocysteine concentrations low because elevated homocysteine is an independent risk factor for cardiovascular disease (4, 132). Hypohomocysteinemia follows the onset of Type 1 diabetes, with hyperhomocysteinemia developing concomitantly with nephropathy as diabetes progresses (143). Increased activity of PEMT and GNMT may lead to increased homocysteine levels. Therefore, it is important to determine why PEMT activity is upregulated in Type 1 diabetes in order to prevent it.

One possible reason is a lack of choline for PC synthesis via the CDP-choline pathway due to increased choline being oxidized to betaine for re-methylation of homocysteine. We tested this theory by supplementing rats with 1% choline or 1% betaine for 10 d prior to the onset of diabetes. PEMT activity remained elevated in streptozotocin-diabetic rats despite 15 d of choline or betaine supplementation. It is possible that the optimal amount of time or supplement was not used. However, it is more likely that PEMT

activity is not regulated by choline or betaine concentrations. There have been several papers suggesting PEMT activity is regulated by glucagon in a cAMP-dependent manner (72, 75, 118). PEMT may be hormonally upregulated to account for the increased need for PC synthesis due to elevated biliary PC secretion (126). Furthermore, studies have shown cAMP analogues inactivate CT by phosphorylation causing its release from the ER and Golgi membranes (144). This effectively inhibits PC synthesis via the CDP-choline pathway. This may be the mechanism used to coordinate PC synthesis via the CDP-choline pathway and the PEMT pathway.

There has also been evidence that hyperglucagonemia upregulates other enzymes in the methyl pathway such as GNMT and CBS (145). Similar to previous results (6), GNMT activity was increased with diabetes, but it was further increased with 1% betaine supplementation. GNMT abundance was not significantly elevated in the diabetic group on the control diet, but was in the diabetic groups supplemented with 1% choline or 1% betaine. GNMT is responsible for optimizing the SAM to SAH ratio (36). The increased flux through the BHMT pathway elevates the amount of SAM in the hepatic one-carbon pool, which would lead to an increased need for GNMT to remove excess SAM. Choline and betaine supplementation would further increase the flux through the BHMT pathway and lead to an even greater surplus of SAM, so GNMT activity would be further elevated.

In agreement with previous findings (6), CBS protein abundance is elevated in Type 1 diabetes regardless of choline or betaine supplementation. One might have expected a decrease in CBS with betaine supplementation due to increased re-methylation of homocysteine by BHMT. However, as mentioned previously there is evidence to show that CBS is transcriptionally regulated by glucagon levels (145).

Supplementation with betaine is currently being explored to prevent or reverse hepatic steatosis (1, 28, 139) and lower plasma total homocysteine levels (1, 30, 146). We found a trend towards increased homocysteine levels with betaine supplementation in the diabetic state. This suggests further studies would need to be done before betaine supplementation can be recommended to diabetics to control their homocysteine levels. Moreover, liver lipids were decreased in all diabetic groups regardless of diet. The exact cause of nonalcoholic steatohepatitis (NASH) is not known, but one mechanism that has been suggested release of cytotoxic free fatty acids from triglycerides that have accumulated in the liver (28). Our data suggests, that at least in the early stages of diabetes, triglycerides are not accumulating in the liver, and betaine supplementation has no effect on liver lipids in the diabetic state. While not all diabetics develop NASH, approximately 75% of people with NASH in the US have diabetes (90, 91). Once again further studies need to be done before betaine supplementation can be safely recommended to diabetics for the prevention of NASH.

In summary, lack of choline for PC synthesis via the CDP-choline pathway does not appear to be the reason for the increased activity of hepatic PEMT in a diabetic state. PEMT activity may be altered by a change in phosphorylation state due to perturbed hormone levels in diabetes. Furthermore, choline and betaine supplementation may actually be detrimental in the diabetic state due to the increased supply of SAM leading to increased homocysteine production. While betaine supplementation has been useful in lowering homocysteine (1, 30, 146) and preventing hepatic steatosis (28) in otherwise healthy humans, more research needs to be done with diabetes before it can be recommended.

Footnotes

[1]This work was funded by the American Diabetes Association

[2]The abbreviations used are: apoB-100, apolipoprotein B-100; BHMT, betaine homocysteine *s*-methyltransferase; CBS, cystathionine β-synthase; CDP, cytidine 5'-diphosphate; CT, CTP:phosphocholine cytidylyltransferase; ER, endoplasmic reticulum; GNMT, glycine *N*-methyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; STZ, streptozotocin; TAG, triacylglycerol; and, VLDL, very low density lipoprotein.

Figure Legends

Figure 4.1. Phosphatidylethanolamine *N*-methyltransferase (PEMT) activity in streptozotocin (STZ)-mediated diabetic rats was unaltered by choline or betaine supplementation. Rats were fed a diet consisting of 0.2% choline bitartrate, 1% choline bitartrate, or 1% betaine anhydrous for 10 d. On d 10 rats were injected with streptozotocin (STZ, 60 mg/kg body weight) or vehicle (10 mmol/L citrate buffer, pH 4.5). On d 15 liver samples were collected and PEMT activity was determined as described under "Materials and Methods". Data are expressed as mean ± SEM (n=4, 5, or 6). Values with an *asterisk* indicates that they were significantly different from control values, $P < 0.05$.

Figure 4.2. Glycine *N*-methyltransferase (GNMT) activity and abundance were increased in streptozotocin (STZ)-mediated diabetic rats and were further increased by 1% betaine supplementation. Rats were fed a diet consisting of 0.2% choline bitartrate, 1% choline bitartrate, or 1% betaine anhydrous for 10 d. On d 10 rats were injected with streptozotocin (STZ, 60 mg/kg body weight) or vehicle (10 mmol/L citrate buffer, pH 4.5). On d 15 liver samples were collected and GNMT activity and protein abundance were determined as described under "Materials and Methods". Data are expressed as mean ± SEM (n=3, 4, 5, or 6). Values denoted with *different letters* indicates that they were significantly different from control values, $P < 0.05$.

Figure 4.3. Cystathionine β-synthase (CBS) protein abundance was increased by streptozotocin (STZ)-mediated diabetes and was unaffected by choline and betaine supplementation. Rats were fed a diet consisting of 0.2% choline bitartrate, 1% choline

bitartrate, or 1% betaine anhydrous for 10 d. On d 10 rats were injected with streptozotocin (STZ, 60 mg/kg body weight) or vehicle (10 mmol/L citrate buffer, pH 4.5). On d 15 liver samples were collected and CBS protein abundance was determined as described under "Materials and Methods". Data are expressed as mean ± SEM (n=3). Bars with an *asterisk* indicates that they were significantly different from control values, $P < 0.05$.

Figure 4.4. Plasma total homocysteine was not significantly different among any treatment group. Rats were fed a diet consisting of 0.2% choline bitartrate, 1% choline bitartrate, or 1% betaine anhydrous for 10 d. On d 10 rats were injected with streptozotocin (STZ, 60 mg/kg body weight) or vehicle (10 mmol/L citrate buffer, pH 4.5). On d 15, blood samples were collected via cardiac puncture, centrifuged, and the plasma fraction used for the determination of homocysteine concentrations by HPLC as described under "Materials and Methods". Data are expressed as mean ± SEM (n=4, 5, or 6).

Tables and Figures

Table 4.1

Liver lipid and plasma triacylglycerol (TAG) concentrations from control and diabetic (STZ, 60 mg/kg body weight) on three different diets; 0.2% choline, 1% choline, and 1% betaine

Values are expressed as mean ± SEM (n=4, 5, or 6). Values within a row with a different letter subscript are significantly different, $P < 0.05$.

	0.2% Choline	0.2% Choline + STZ	1% Choline + STZ	1% Betaine + STZ
Liver Lipids *(mg/g liver)*	38.25 ± 1.28^{a}	32.78 ± 0.23^{b}	30.13 ± 0.76^{b}	31.89 ± 1.46^{b}
Plasma TAG *(mmol/L)*	0.82 ± 0.12	2.03 ± 0.86	1.77 ± 0.62	2.61 ± 1.06

Figure 4.1

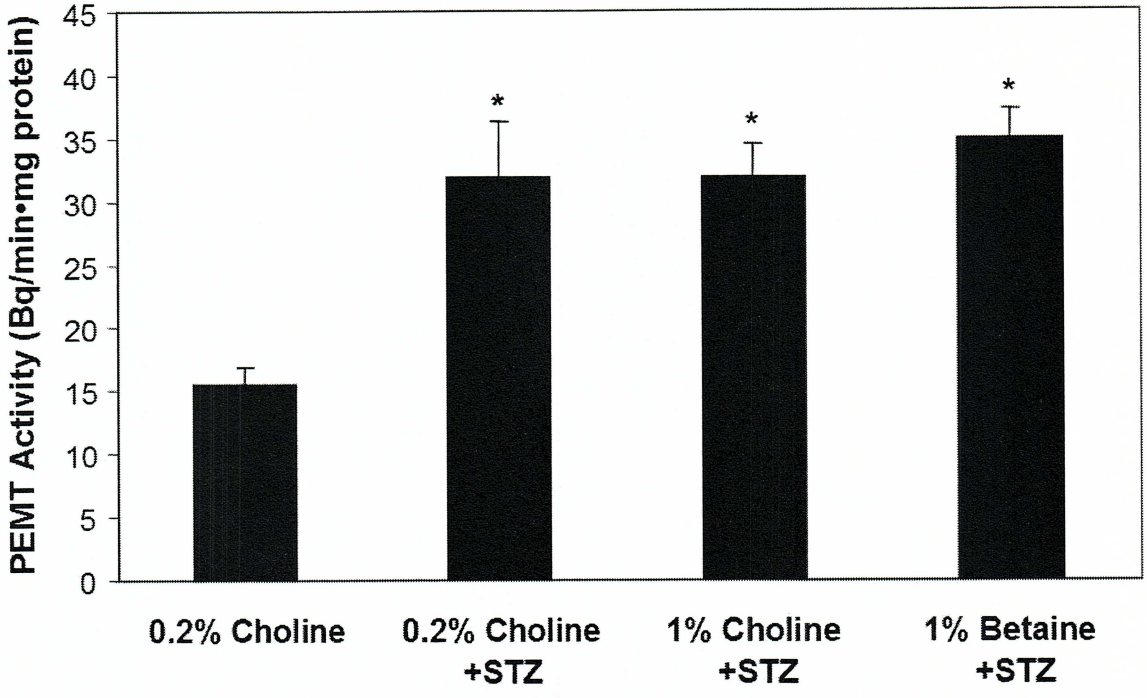

PEMT Activity (Bq/min•mg protein)
45
40
35
30
25
20
15
10
5
0
*
*
*
0.2% Choline
0.2% Choline +STZ
1% Choline +STZ
1% Betaine +STZ

Figure 4.2

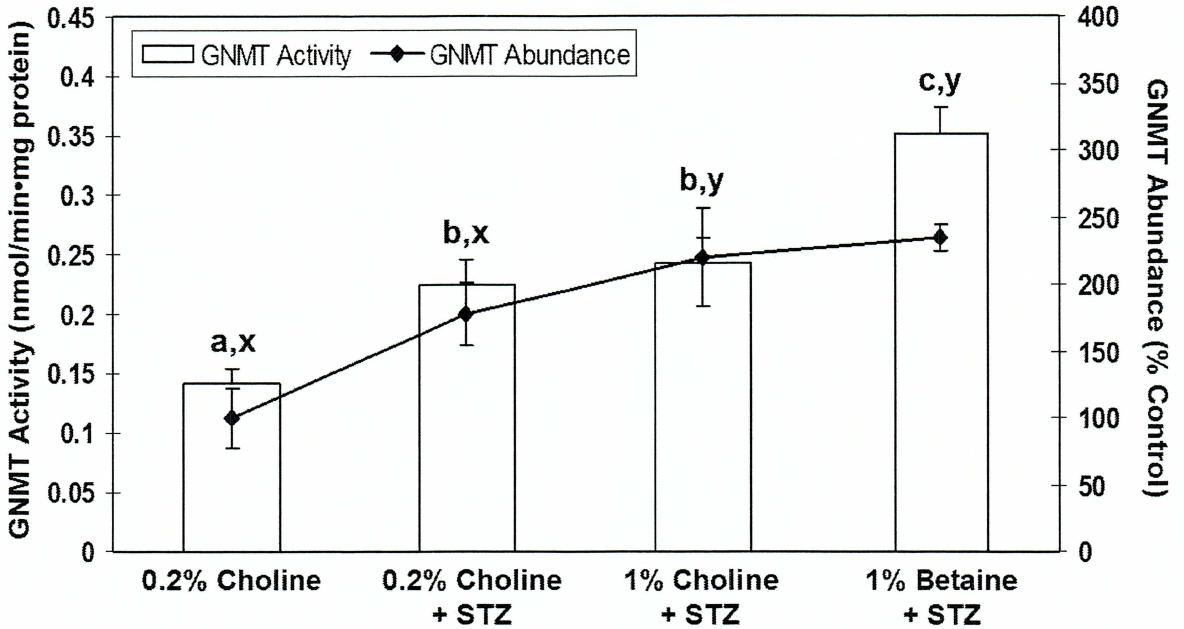

GNMT Activity
GNMT Abundance
GNMT Activity (nmol/min•mg protein)
GNMT Abundance (% Control)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
400
350
300
250
200
150
100
50
0
a,x
b,x
b,y
c,y
0.2% Choline
0.2% Choline + STZ
1% Choline + STZ
1% Betaine + STZ

Figure 4.3

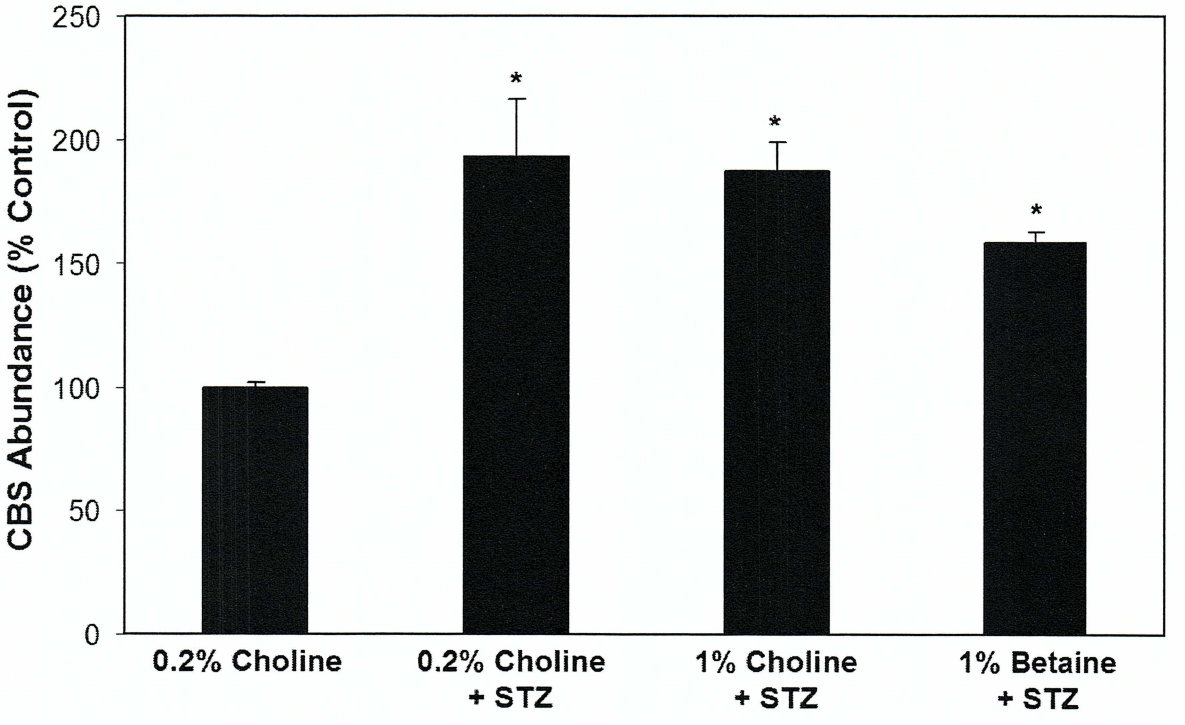
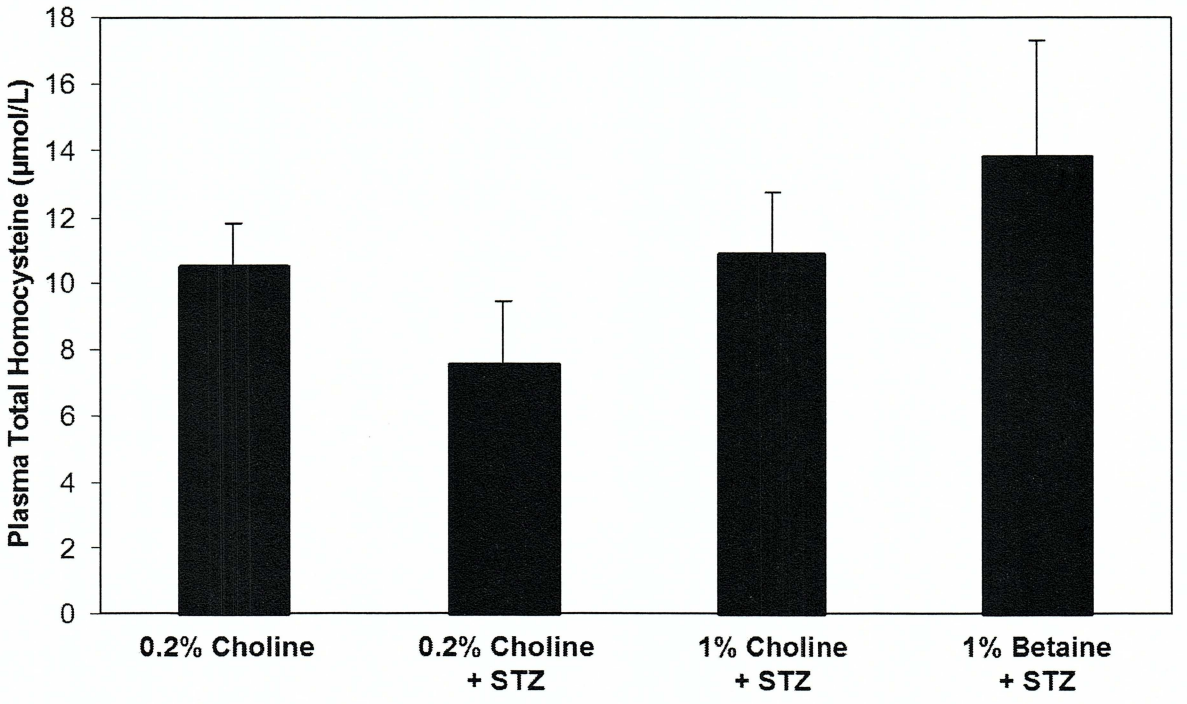

CBS Abundance (% Control)
250
200
150
100
50
0
*
*
*
0.2% Choline
0.2% Choline + STZ
1% Choline + STZ
1% Betaine + STZ

Figure 4.4


Plasma Total Homocysteine (µmol/L)
18
16
14
12
10
8
6
4
2
0
0.2% Choline
0.2% Choline + STZ
1% Choline + STZ
1% Betaine + STZ

CHAPTER 5. GENERAL CONCLUSIONS

Phosphatidylcholine (PC) is essential for many actions in the body including cell membrane integrity, bile, VLDL secretion, and cell signaling. There are two major pathways to synthesize PC in the body; the CDP-choline pathway and the PEMT pathway. The CDP-choline pathway requires choline, whereas the PEMT pathway requires methyl groups. Under normal conditions, PEMT synthesizes approximately 30% of the hepatic PC (7-9). This makes PEMT a key enzyme in the methyl group metabolism pathway and is considered by some to be an important regulator of homocysteine (10, 43).

There are several factors that modulate hepatic methyl group metabolism. In the beginning of this thesis we focused on diabetes and administration of retinoids. While retinoids affect other enzymes involved in methyl group metabolism, it appeared they had no effect on PEMT activity. However, streptozotocin-diabetes consistently and rapidly caused a 2-fold induction of PEMT activity. There is evidence that diabetics have increase biliary PC secretion, which would require increased PC synthesis (126). Also, PC synthesis, particularly via phospholipid methylation, is important in maintaining VLDL secretion (77, 127, 137, 138, 147) to prevent fatty liver. We did not directly investigate the affects of diabetes on the CDP-choline pathway, so at this time it is unclear if PEMT activity is increased to meet an increased demand for PC synthesis or if it is in response to decreased PC synthesis by the CDP-choline pathway.

To try to elucidate if the increase in PEMT activity seen with diabetes is in response to a decrease in PC synthesis by the CDP-choline pathway, we tried supplementing diabetic rats with choline or betaine. It was reasoned that the increase in BHMT activity seen with diabetes may be resulting in an increased requirement for choline or betaine. It was found

that choline and betaine supplementation had no effect on PEMT activity in the diabetic state. However, betaine supplementation did further increase the activity of another methyl transferase, GNMT, and it seemed to attenuate the decrease in plasma total homocysteine typically seen with the onset of diabetes. Betaine supplementation is currently used to lower plasma homocysteine levels in individuals with hyperhomocysteinemia and is being studied to prevent or reverse hepatic steatosis (1, 28, 30, 139, 146, 148-150). These two diseases are considered to be major complications of diabetes. It appears that the preliminary work from our study shows that betaine supplementation may not be an effective treatment in diabetics for hyperhomocysteinemia and hepatic steatosis and may in fact be detrimental to their health.

Another conclusion that can be drawn from this final study is that it is not a lack of choline for the CDP-choline pathway that is driving the increase in PEMT activity seen with diabetes. This further supports other studies suggesting the PEMT activity is hormonally regulated by glucagon and that elevated levels of glucagon typically seen with diabetes is the cause for increased PEMT activity (75, 118, 145). They also tested the affect of cAMP analogues and found similar increases in PEMT activity as those seen with glucagon treatment (75). This led them to conclude there is either increased or decreased phosphorylation of the enzyme in the diabetic state leading to its increased activity. This is an avenue that needs to be explored further. If it is the elevated glucagon that is causing the alterations, then it may be necessary in the future for diabetics to monitor their glucagon levels as well as their insulin levels.

There is much work that needs to be done in this area to determine the mechanism for how diabetes alters phospholipid methylation, how this affects PC synthesis, and what

overall consequences does this have on the body. This work will require primary hepatocytes cell cultures to determine the direct signals causing increased PEMT activity. It will probably also be necessary to look at PC synthesized by the CDP-choline pathway in the diabetic state. And finally, it will be necessary to determine how this affects bile secretion, VLDL secretion, cell membrane integrity, and homocysteine levels. Also, betaine supplementation in diabetic rats should be studied for a longer time period and at different levels to determine if there are any detrimental affects of supplementation. Until longer term studies have been done, it should not be recommended that diabetics supplement their diets to lower homocysteine or treat hepatic steatosis. It will also be necessary to determine if insulin injections to diabetic rats can reverse some of the alterations found in order to help translate these findings to human subjects. Once the consequences of diabetes on phospholipid synthesis and usage are better understood, we can then move forward with better treatments.

APPENDIX: EFFECTS OF DIABETES AND FOLATE STATUS ON PHOSPHATIDYLETHANOLAMINE N-METHYLTRANSFERASE AND GUANIDINOACETATE N-METHYLTRANSFERASE[1]

Introduction

Folate status is highly important in regulation of methyl group metabolism. Folate and betaine are responsible for donating one-carbon units to homocysteine in order to regenerate methionine. Folate deficiency can lead to decreased levels of *S*-adenosylmethionine (SAM) and increased levels of *S*-adenosylhomocysteine (SAH), causing inhibition of methyltransferases. Type 1 diabetes has also been shown to alter the activities of many methyltransferases including GNMT and BHMT (6). This study was conducted to determine how folate status combined with diabetes affect the activities of phosphatidylethanolamine *N*-methyltransferase (PEMT) and guanidinoacetate *N*-methyltransferase (GAMT).

Materials and Methods

Animals

All experiments involving animals were approved by and conducted in accordance with the Iowa State University Laboratory Animal Resources guidelines. Thirty-six male Sprague-Dawley (Harlan Sprague-Dawley, Indianapolis, IN) rats (50-74 g) were housed in separate cages with a 12-h light: dark cycle and were allowed access to food and water *ad libitum*. Rats were randomly assigned to treatment groups and fed an amino-acid defined diet (Harlan Teklad, Madison, WI) containing either 0 (folate-deficient, FD), 2 (folate-adequate, F), or 8 ppm folate (folate-supplemented, FS). After 30 d, half of the rats in each dietary folate group received a single intraperitoneal injection of streptozotocin (STZ, 60

mg/kg body weight) or vehicle (10 mM citrate buffer, pH 4.5). On day 35, rats were anesthetized with ketamine: xylazine (90: 10 mg/kg body weight). The liver was snap-frozen in liquid nitrogen and stored at -70°C until determination of PEMT and GAMT activities.

Determination of PEMT and GAMT activity

Approximately 1 g of frozen liver was homogenized in 4 volumes of ice-cold 10 mM Tris HCl (pH 7.4) containing 0.25 M sucrose. The homogenate was centrifuged at 16,000 × g for 20 min at 4°C and 1-2 mL of the resulting supernatant was centrifuged at 105,000 × g for 60 min at 4°C. The supernatant was discarded and the microsomal pellet was resuspended in 400 µl of 0.25 M sucrose. The microsomal fraction was stored at -70°C until analysis. Total soluble protein concentrations were determined by the method of Bradford (119). PEMT activity 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.* (120) with modifications. Briefly, the reaction mixture contained 10 mM HEPES (pH 7.3), 4 mM dithiothreitol, 5 mM $MgCl_2$, 0.1 mM SAM, 2 µCi S-adenosyl-L-[methyl-^{3}H]methionine, and 750 µg protein in a final volume of 550 µL. The reaction was initiated by adding 75 µL microsomal protein (10 µg/µL) to the assay mixture and incubated in a 37°C water bath for 10 min. The reaction was terminated by pipeting 100 µL of the assay mixture into 2 mL chloroform: methanol: 2 N HCl (6:3:1, v:v:v) in duplicate. The chloroform phase was washed three times with 1 mL 0.5 M KCl in 50% methanol, transferred to a glass scintillation vial, and allowed to dry at room temperature. The lipid fraction was reconstituted in 5 mL Scintverse® (Fisher Scientific, Pittsburgh, PA) scintillation fluid and the radioactivity was determined by liquid scintillation counting. GAMT activity was analyzed using the method of Xue and Snoswell (98) with modifications. Briefly, 500 µg

protein samples were incubated for 20 min at 37°C in a 100 µL reaction volume containing 50 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 2 mM guanidoacetate, and 0.1 µCi S-adenosyl-L-[methyl-^{3}H]methionine. The reaction was terminated by the addition of 100 µL of 10% trichloroacetic acid and buffered by the addition of 400 µL 0.2 M Tris. Reaction samples were applied to a 1 x 3 cm column containing Bio-Rad AG-50-X4 resin (NH_4 form, 50-100 mesh) and eluted with three 1 mL volumes of deionized H_2O directly into scintillation vials for liquid scintillation counting.

Results

PEMT activity was increased with diabetes, but folate status was without effect. Phosphatidylethanolamine *N*-methyltransferase (PEMT) activity was increased by 87-118% in all STZ treatment groups versus their controls on the same diets (**Table A.1**). There were no differences among the diet groups in PEMT activity.

GAMT was decreased with diabetes. In all STZ treatment groups, GAMT activity was significantly reduced 40-59% versus their respective controls (**Table A.1**). The folate-sufficient group showed a modestly increased activity of GAMT as compared to the folate-deficient and the folate-supplemented groups.

Discussion

The increase in PEMT activity in the STZ-diabetic groups was consistent to what was shown in our previous studies. The lack of effect by folate-status was also consistent with the results seen with choline and betaine supplementation. This is simply further evidence that the increase in PEMT activity does not seem to be substrate, but more likely hormonally regulated.

GAMT activity in diabetes may be the result of inhibition by elevated levels of SAH. Another possibility is SAM is preferentially shuttled to PEMT to synthesize more PC leaving less SAM for GAMT and consequently a decrease in activity. Another possibility is GAMT is hormonally regulated. Increased levels of glucagon in the diabetic state is a signal the body is in starvation mode. GAMT activity and possibly expression may be decreased to conserve methyl groups for more important reactions such as PC synthesis. This could mean diabetics have decreased creatine production.

Folate-deficiency and folate-supplementation could lead to less than optimal levels of SAM and SAH. GAMT activity in the folate-sufficient non-diabetic group may have been increased due an optimized ratio of SAM:SAH.

In summary, PEMT activity in this study mirrored the results of other studies and provided more evidence that PEMT activity is probably not upregulated in diabetes due to a change in the size of the hepatic one-carbon pool. The decrease in GAMT activity may be partially responsible for the increase in GNMT activity seen in other studies. With the decrease in GAMT activity in the diabetic state, it could be a concern that diabetics are not able to synthesize enough creatine. Creatine is an essential compound for muscle function, especially during exercise. Creatine supplementation may be necessary in the diabetic state.

Footnotes

[1]This study was done on rat liver samples obtained from a study done by Kristin Nieman

Tables and Figures

Table A.1

Hepatic activity of phosphatidylethanolamine *N*-methyltransferase (PEMT) and guanidinoacetate *N*-methyltransferase (GAMT) in rats treated with streptozotocin (STZ) and fed either 0 (FD), 2 (F), or 8 (FS) ppm dietary folate

Values are expressed as mean ± S.E. ($n = 6$). Values within a column with different letter superscript are significantly different, $p < 0.05$.

	PEMT	**GAMT**
	Bq/min•mg protein	*pmol/min•mg protein*
FD	26.1 ± 1.1^{a}	0.30 ± 0.02^{a}
FD + STZ	50.1 ± 10.3^{b}	0.15 ± 0.03^{b}
F	28.3 ± 2.0^{a}	0.44 ± 0.04^{c}
F + STZ	52.8 ± 5.8^{b}	0.18 ± 0.05^{b}
FS	28.4 ± 2.4^{a}	0.30 ± 0.01^{a}
FS + STZ	62.0 ± 5.6^{b}	0.18 ± 0.02^{b}

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