

**Nutritional and hormonal modulation of diabetes-perturbed folate, homocysteine,
and methyl group metabolism**

by

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LIST OF ABBREVIATIONS

5-CH ₃ THF	5-methyltetrahydrofolate
5,10-CH ₂ THF	5,10-methylenetetrahydrofolate
10-CHO-THF	10-formyltetrahydrofolate
13- <i>cis</i> RA	13- <i>cis</i> -retinoic acid
ApoE	apolipoprotein E
BHMT	betaine-homocysteine <i>S</i> -methyltransferase
<i>b.i.d.</i>	<i>bis in die</i> (twice daily)
CBS	cystathionine β-synthase
CRA	13- <i>cis</i> -retinoic acid
CVD	cardiovascular disease
D	vitamin D- supplemented
DFE	dietary folate equivalent
DMG	dimethylglycine
DNMTs	DNA methyltransferases
DRI	dietary reference intake
D+STZ	vitamin D-supplemented diabetic
dUMP	deoxyuridine monophosphate
EAR	estimated average requirement
F	folate-adequate diet
FD	folate-deficient diet
FS	folate-supplemented diet
GAMT	guanidinoacetate methyltransferase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GNMT	glycine <i>N</i> -methyltransferase
Hcy	homocysteine
HPLC	high performance liquid chromatography
INS	insulin
MAT	methionine adenosyltransferase

MS	methionine synthase
MTHFR	5,10-methylenetetrahydrofolate reductase
NOD mice	non-obese diabetic mice
NTDs	neural tube defects
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine <i>N</i> -methyltransferase
RA	all- <i>trans</i> -retinoic acid
RDA	recommended dietary allowance
SAM	<i>S</i> -adenosylmethionine
SAH	<i>S</i> -adenosylhomocysteine
SAHH	<i>S</i> -adenosylhomocysteine hydrolase
SHMT	serine hydroxymethyltransferase
STZ	streptozotocin
THF	tetrahydrofolate
TS	thymidylate synthase
UL	upper limit
ZDF rat	Zucker diabetic fatty rat

ABSTRACT

Folate, homocysteine, and methyl group metabolism mutually function to provide methyl groups for numerous biosynthetic- and modification-type reactions necessary for optimal health. Methyl groups from these pathways are utilized in the production of important compounds such as nucleic acids, protein, and phospholipids. Thus it is vital to understand the factors that both disrupt and maintain their delicate balance to prevent disease. Disruption of these metabolic pathways is associated with several pathologies including carcinogenesis, birth defects, and vascular disease. Moreover, the disruption of these metabolic pathways may be a link between chronic disease and complications of that disease. Both nutritional and hormonal factors have been shown to modulate these interrelated metabolic pathways, commonly altering enzymes involved.

Diabetes mellitus is estimated to affect approximately 21 million Americans, constituting nearly seven percent of the population. Roughly one-third of this group is unaware that they even have diabetes, which is a major cause of morbidity and mortality in this country. Both type 1 and type 2 diabetes, characterized by elevated blood glucose concentrations, lack of or insensitivity to insulin, and elevated counter-regulatory hormones to insulin (*i.e.* glucagon and glucocorticoids), have been shown to disrupt folate, homocysteine and methyl group metabolism.

Homocysteine pools, the non-protein forming amino acid synthesized and catabolized by these pathways, were also disrupted in diabetics. Hypohomocysteinemia is commonly reported in diabetics without renal complications; however as renal function deteriorates in chronic diabetes, hyperhomocysteinemia results. Furthermore, hyperhomocysteinemia is considered an independent risk factor for vascular disease. It is estimated that heart disease and stroke account for 65 percent of deaths in diabetics. Glycine *N*-methyltransferase (GNMT), the enzyme responsible for controlling methyl group supply and utilization, is also disrupted in diabetes, leading to depletion of essential methyl groups. Activity of this enzyme is allosterically regulated by the folate coenzyme, 5-methyltetrahydrofolate.

Vitamin D status has been implicated in diabetic pathogenesis. In particular, vitamin D deficiency has been shown to increase the risk of developing type 1 diabetes and supplementation may reduce this risk. Further, vitamin D deficiency has been associated

with impaired insulin secretion and glucose intolerance, but ameliorated by repletion. As a result, this research was aimed at determining if supplemental folate, supplemental vitamin D, and insulin repletion could prevent perturbations in homocysteine and methyl group metabolism induced by a type 1 diabetic state.

Insulin administration restored enzyme activity, protein abundance, and homocysteine concentrations in diabetic rats. Indicating perturbations in folate, homocysteine and methyl group metabolism do result from a diabetic state and are not the result of streptozotocin toxicity. GNMT, perturbed by a diabetic state, was attenuated in diets containing adequate or supplemental folate, likely due to posttranslational modification. Although these results would suggest that adequate folate status has a positive effect under diabetic conditions, supplemental folate did not confer any added benefit at the level tested. Further, betaine homocysteine *S*-methyltransferase (BHMT), the folate-independent remethylation enzyme, and GNMT mRNA were induced in diabetic animals indicating modification in a diabetic state is possibly at the level of transcription. Vitamin D supplementation restored homocysteine pools disrupted in a diabetic state potentially as a result of increased homocysteine production. This research indicates folate, vitamin D, and insulin may be involved in modulating homocysteine and methyl group metabolism in a diabetic state. Ultimately it will be critical in future work to more clearly determine and evaluate the factors that both regulate and alleviate diabetes-perturbed homocysteine and methyl group metabolism for optimal health and prevention of complications associated with diabetes.

CHAPTER 1: GENERAL INTRODUCTION

Thesis Organization

The contents of this thesis are organized into several chapters. Chapter 1 is an introduction giving the reader an idea of the research questions the author focused on. Chapter 2 is broad review of the literature, including information that is pertinent to the author's research in folate, homocysteine, and methyl group metabolism. Chapter 3 examines the role of insulin in modulating methyl group metabolism altered by a diabetic state. Modified portions of this chapter were published in the *Journal of Nutrition* and the *Federation of American Societies for Experimental Biology Journal*. Chapter 4 contains research that was aimed at determining the role of supplemental folate in diabetes-perturbed homocysteine and methyl group metabolism. This chapter was modified from a paper published in the *American Journal of Physiology-Endocrinology and Metabolism* and an abstract published in the *Federation of American Societies for Experimental Biology Journal*. Chapter 5 describes a study that was conducted to determine the role of vitamin D in homocysteine metabolism, disrupted by diabetes. A small modified portion of this chapter was also published in the *Federation of American Societies for Experimental Biology Journal*. General conclusions and ideas for future research are incorporated into chapter 6 including a list of the literature cited throughout the entire document.

Description of the Research Questions

The interrelated folate, methyl group, and homocysteine pathways function to carry and activate one-carbon units in the synthesis and modification of essential biological compounds. Specifically, these mechanisms are vital in the production of nucleic acids, proteins, and phospholipids. Therefore interruption of these pathways can result in a number of diseases including cancer, vascular disease, and birth defects.

A number of factors have been identified as having the ability to alter folate, methyl group, and homocysteine metabolism. A diabetic state has been identified as one of these factors and the focus of this research. Type 1 and type 2 diabetes, characterized by elevated blood glucose, lack of or insensitivity to insulin, and elevated counter-regulatory hormones to insulin (*i.e.* glucagon and glucocorticoids), have been shown to disrupt folate, homocysteine, and methyl group metabolism. Specifically, homocysteine pools, the non-protein forming

amino acid synthesized and catabolized by these pathways, are also disrupted in diabetics depending on renal function. Hypohomocysteinemia is commonly reported in diabetics without renal complications; however as renal function deteriorates in chronic diabetes elevated homocysteine concentrations result. The enzyme responsible for controlling methyl group supply, glycine *N*-methyltransferase (GNMT), is also disrupted in diabetes leading to depletion of essential methyl groups. GNMT activity is allosterically regulated by the folate coenzyme, 5-methyltetrahydrofolate.

Several studies have also reported that compounds capable of simulating a gluconeogenic state, including glucagon and glucocorticoids, also have the ability to modify homocysteine and methyl group metabolism. Rats treated with glucagon exhibited elevated activities of GNMT and the enzyme that initiates homocysteine catabolism cystathionine β -synthase (CBS). Decreased homocysteine concentrations have also been reported indicating increased flux through the transsulfuration pathway. Administration of the glucocorticoid dexamethasone has been shown to induce and activate GNMT in cell culture and animal models, as well. Studies conducted in both streptozotocin-induced diabetic rats and type 2 diabetic rat models, have also revealed similar modifications in one-carbon metabolism.

Further, vitamin D status has been implicated in diabetic pathogenesis. Vitamin D deficiency has been shown to increase the risk of developing type 1 diabetes in infants and mice models of type 1 diabetes; while supplementation may reduce this risk. Vitamin D deficiency has been associated with impaired insulin secretion and glucose intolerance, but ameliorated by repletion. As a result, the work described in this document was aimed at determining if supplemental folate, supplemental vitamin D, and insulin repletion could prevent perturbations in homocysteine and methyl group metabolism induced by type 1 diabetes.

CHAPTER 2: A REVIEW OF THE LITERATURE

Folate Metabolism

Structure and Function of Folate Compounds

Structure

Folate, a generic term for a family of essential water soluble B-vitamins, functions primarily to accept and donate one-carbon units in vital metabolic pathways throughout the body (16, 158). This family of vitamins, also known by the chemical name pteroylglutamic acid, contains both natural and synthetic forms all of which are similar in structure. The basic structure of folate moieties consists of a pteridine ring, p-aminobenzoic acid, and one or more glutamic acid residues linked by γ -carboxyl peptide bonds (**Figure 2.1A**). Folate forms differ by altering the structure in three locations: modifying the reduction state of the pteridine ring, adding one-carbon units at the N₅ and/or N₁₀ binding sites (**Figure 2.1B**), or varying the number of glutamic acid residues attached. Naturally occurring folate is typically

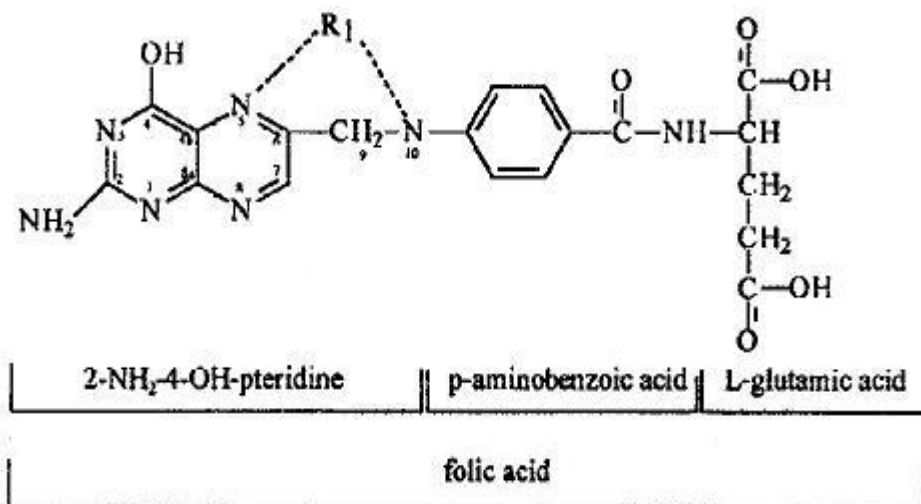


Figure 2.1A. Basic structure of folate molecules (modified from (284)).

polyglutamated containing between three and seven glutamyl residues and exists most commonly as 5-methyltetrahydrofolate (5-CH₃THF) and 10-formyltetrahydrofolate (10-CHO-THF). The synthetic form of folate, folic acid used in supplements and fortified foods, is monoglutamated (284).

Function

The donation of one-carbon units is essential in the production of nucleic acids (DNA and RNA), proteins, as well as in the metabolism of amino acids. These one-carbon units exist at various oxidation levels, as seen in figure 2.1B, and the reactions they are involved in are collectively known as folate-dependent one-carbon metabolism (289). The reduced folate derivative, tetrahydrofolate (THF), accepts one-carbon units at the oxidation level of formate

R ₁	N ³	N ¹⁰		oxidation state of one-carbon moiety
	—CH ₃	—H	5-methyl	
	—CH ₂ —	—CH ₂ —	5,10-methylene	
	—CH=	—CH=	5,10-methenyl	
	—CHNH	—H	5-formimino	
	—CHO	—H	5-formyl	
	—H	—CHO	10-formyl	
				reduced ↑ oxidized

Figure 2.1B. Folate derivatives (modified from (284)).

from histidine degradation, or at the oxidation level of formaldehyde from the 3-carbon of serine (248). However, one-carbon units can also be derived from glycine, dimethylglycine (DMG), and *N*-methylglycine (sarcosine) (284). Specifically folate, in the form of 10-CHO-THF, is needed to donate carbon 2 and 8 in the purine component of DNA (**Figure 2.2**). The folate derivative 5,10-methylenetetrahydrofolate (5,10-CH₂THF) is involved in the biosynthesis of deoxythymidine monophosphate, from deoxyuridine monophosphate, a pyrimidine building block of DNA (241, 248, 289). Aside from the role of folate in the biosynthesis of nucleic acids, one-carbon units as CH₃ provided by folate are used in over fifty methyltransferase-catalyzed reactions (56, 284) (**Figure 2.3**).

Folate Utilization

Digestion and Absorption

Mammals are unable to synthesize folates *de novo*, making it necessary to attain folate

compounds through consumption (247). The polyglutamated chain of naturally occurring folates must be removed before the vitamin is absorbed. Excess glutamate residues are hydrolyzed by the enzyme γ -glutamyl hydrolase in brush border mucosal cells of the proximal small intestine, and in turn this monoglutamated form is absorbed (181). Typically if the folate compound is not already in the form of 5-CH₃THF, it will be converted to this form and transported through the intestinal mucosa cell (239). The monoglutamated synthetic form of folate is readily absorbed, primarily in the jejunum.

Transport and Storage

Following absorption, folate passes through the mucosal cell and is taken up by the liver

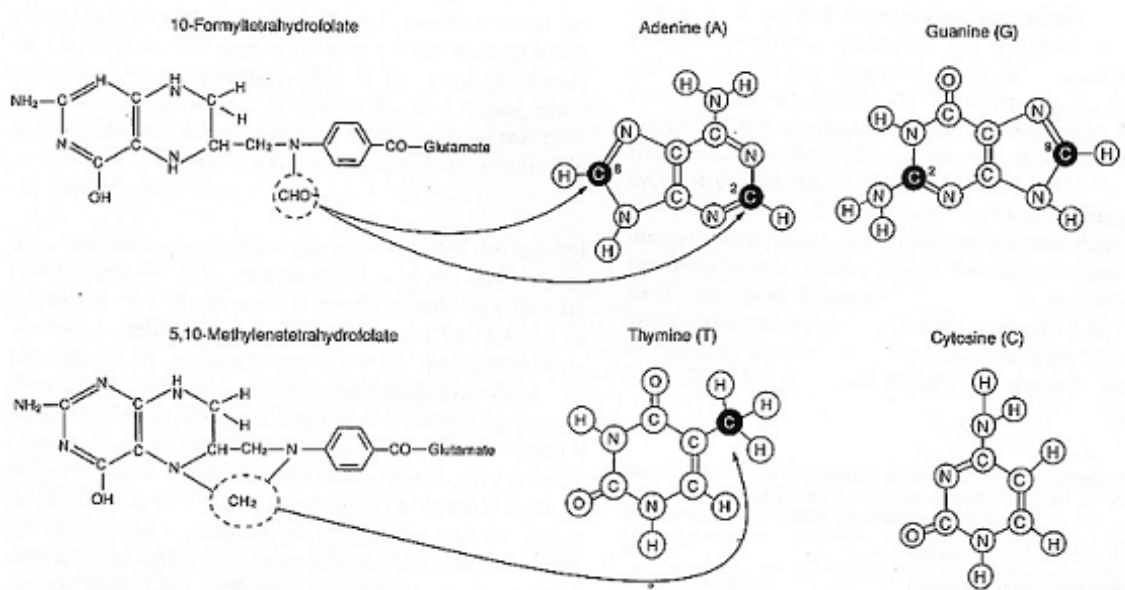


Figure 2.2. The role folate in purine and pyrimidine biosynthesis (from (241)).

via portal circulation (247). In the liver, folate remains in the form 5-CH₃-THF or is otherwise converted to 10-CHO-THF and transported to peripheral tissue (181). The transport of folate into cells can be accomplished by one of three mechanisms: (i) unidirectional transport by membrane-bound folate-binding proteins/receptors, (ii) bidirectional internalization by carrier-mediated reduced folate carriers, (iii) or by passive

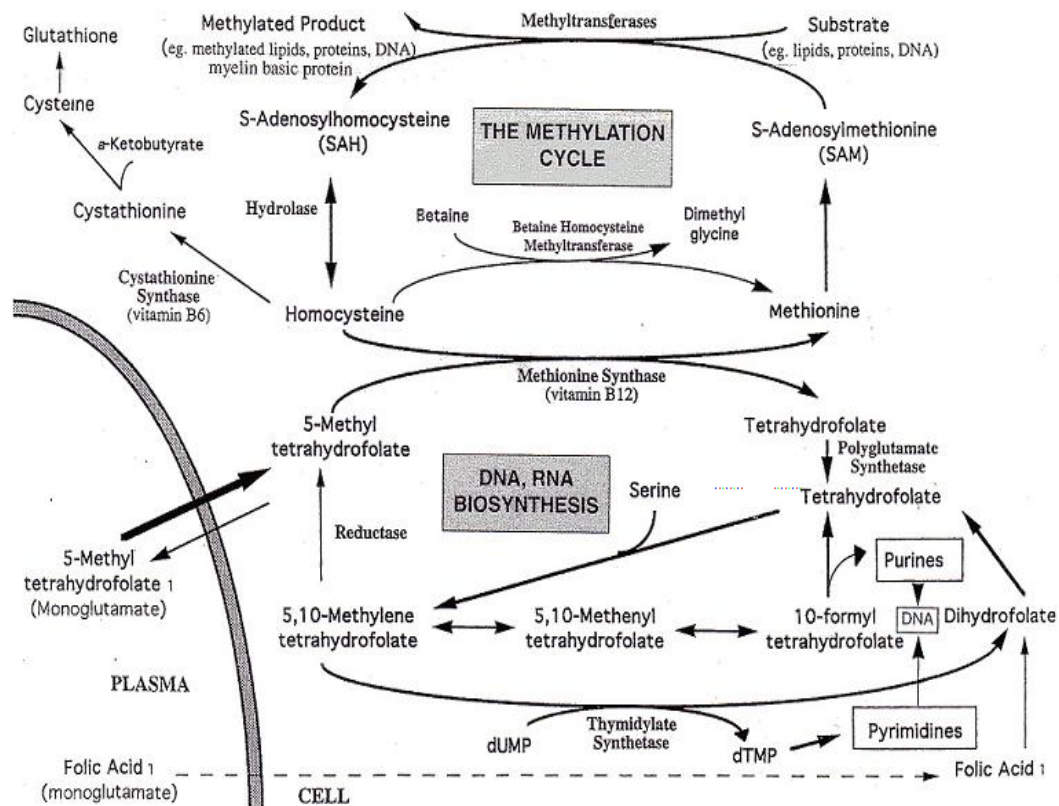


Figure 2.3. Folate and one-carbon metabolism (from Scott and Weir 1998)

diffusion (252, 265). Once folate enters the cell the enzyme methionine synthase (MS) converts 5-CH₃THF to THF the favored substrate of the enzyme, folylpolyglutamate synthase. The activity of this enzyme, necessary to retain folate within the cell, results in the polyglutamylation of THF and is present in both the mitochondria and cytoplasm (158).

Polyglutamylated folate is compartmentalized between the cytoplasm and the mitochondria (58). Most folate in the cell is protein-bound and usually remains in their respective compartment; however the products that result from folate compounds are easily transferred between the cytoplasm and mitochondria (154). At any time cellular concentrations of free folate are negligible due to tight-binding proteins, which also limits intracellular folate accumulation when saturated (172). The body typically stores between 20 and 70 mg of folate (100, 263), half of which is found in the liver in the form of 5-CH₃THF (105, 106).

Degradation and Excretion

Protein-bound folates are stable compared to free-folate, which is often depleted in the cell due to its instability. There are three mechanisms that are thought to cause the turnover of folates, specifically unbound folate: exit of folate molecules from lack of polyglutamylation, folate polyglutamates are hydrolyzed to monoglutamates by γ -glutamylhydrolase and exit the cell, or folate catabolism (265). A majority of folate excretion is in feces via bile due to enterohepatic circulation most of which is reabsorbed in the intestine. However, a small portion of excreted folate can be found in urine following glomerular filtration and reabsorption of some of the folate (105). Degradation of folate generates pteridines and acetaminobenzoylglutamate.

Dietary Requirements

Sources of Folate

Currently recommendations for folate intake are focused on reducing the risk of chronic disease, which means maintaining optimal one-carbon metabolism (31). Folates are present in most natural foods; fruits and vegetables being the richest sources. Specifically, spinach, brussel sprouts, potatoes, oranges, beans, and liver, are excellent sources of folate (15, 284). However, these natural forms of folate are particularly susceptible to oxidative damage, and it is estimated that 50-95% of the folate is destroyed by cooking excessively (105). There are also several states in the body that can result in altered folate status and increased catabolism and in turn increases folate requirement such as pregnancy, oral contraceptive or anticonvulsant use, cancer, or chronic alcohol consumption (265). It is because of this potential for folate deficiency and the risk of congenital defects as well as chronic disease associated with deficiency that the U.S. Food and Drug Administration made it mandatory for enriched grain products to include folic acid (2). This fortification has led to a decline in the prevalence of folate deficiency-related pathologies specifically neural tube defects (113).

Recommended Intake

Folate dietary requirements are expressed as recommended dietary allowances (RDA) which is derived from the estimated average requirement (EAR) as well as adjusting to meet the requirements for approximately 98% of the population. The EAR is defined as the amount needed to meet 50% of the population (16). Given that synthetic folate is much more

readily available than dietary folate, dietary reference intakes (DRIs) for folate are expressed as dietary folate equivalents (DFE). One DFE is equivalent to 1 μg of food folate, 0.6 μg of synthetic folate from fortified food or a supplement with food, or 0.5 μg of a supplement on an empty stomach (1).

RDAs vary depending on age and physiological state. Males and females older than 14 years of age are recommended to take in 400 μg DFE per day. The RDA declines for children less than 14 years old to between 65 and 80 μg DFE per day in infants up to 12 months old. Folate requirements increase in the case of pregnancy and lactation at 600 and 500 μg DFE per day, respectively (**Table 2.1**). This elevated requirement is due to the increased need for folate in cell division and increased catabolism of folate during pregnancy, as well as the loss of folate in daily lactation following pregnancy (15). There are also upper limits (UL) specified for each age group, meaning the maximum daily intake without inducing adverse effects. In children up to 8 years old the UL is 300-400 μg DFE per day and 600-800 μg DFE per day in males and females up to the age of 18 years old. The UL for adults above 19 years old increases to 1,000 μg DFE per day in both males and females regardless of pregnancy and lactation (1).

Methyl Group Metabolism

Functions and Components of the Metabolic Pathway

Transmethylation

Methyl group metabolism functions in activating methyl groups required for numerous methylation reactions necessary for normal growth and development. Methyl group metabolism begins with the set of reactions collectively called transmethylation which relies on a supply of the essential sulfur-containing amino acid, methionine (78). Methionine is converted to its activated form, *S*-adenosylmethionine (SAM) following adenylation. This reaction is catalyzed by the enzyme methionine adenosyltransferase (MAT) at the expense of ATP (39, 52, 153). At this point SAM, the primary methyl donor in most mammals can donate activated methyl groups to many methylation reactions in the modification and synthesis of essential molecules, resulting in *S*-adenosylhomocysteine (SAH) (169, 191). The formation of creatine from guanidinoacetate by guanidinoacetate methyltransferase (GAMT) constitutes a major use of methyl groups (194, 260). However, it has been

Table 2.1*Folate dietary reference intakes¹*

Group	Adequate intake (µg of DFE/d)	Recommended dietary allowance (µg of DFE/d)
infants		
0-6 months	65	
7-12 months	80	
children and adolescents²		
1-3 years		150
4-8 years		200
9-13 years		300
4-18 years		400
adults²		
≥ 19 years		400
pregnant women		
all ages		600
lactating women		
all ages		500

¹ modified from Bailey and Gregory 1999² male and female

suggested that phosphatidylethanolamine *N*-methyltransferase (PEMT) is the primary consumer of methyl groups from SAM in the synthesis of phosphatidylcholine (PC) from phosphatidylethanolamine (PE), thus being the primary contributor to homocysteine production (126, 256). PEMT accounts for 20-40% of hepatic PC synthesis (267), but can increase to compensate for PC production as seen in the CTP:phosphocholine cytidyltransferase- α knockout mouse (126). These animals are incapable of synthesizing PC through the CDP-choline pathway, the primary pathway for PC production. PC is necessary for phospholipids, lipoproteins, and bile, as well as intracellular communication (312). Most SAM-dependent transmethylation reactions are inhibited by SAH, therefore it essential to regulate the SAM to SAH ratio as well as remove SAH to preserve methyl groups (41).

The folate-binding protein, glycine *N*-methyltransferase (GNMT), is believed to function in regulating the availability of SAM (60, 310). GNMT is found in the liver, pancreas, and kidney, and comprises approximately 1 to 3 % of all soluble hepatic cytosolic protein (135, 205). GNMT was found to be most abundant in the periportal region of the liver, the

proximal convoluted tubules of the kidney, and the exocrine tissue of the pancreas (310). This methyltransferase converts SAM to SAH, utilizing glycine and forming sarcosine, which currently has no known metabolic role (103, 135). GNMT optimizes the SAM:SAH ratio, which is an indicator of transmethylation potential, given SAH is a potent inhibitor of most other SAM-dependent methyltransferases (291, 310). This regulatory role has been supported by the GNMT knockout mouse model. Hepatic GNMT activity and expression were completely eliminated in homozygous animals and the SAM:SAH ratio increased by over 100-fold (162). In further support of the regulatory role of GNMT, activity and expression of the enzyme were induced in the brain of PEMT knockout mice (318). The activities of all other methyltransferases measured in this study were decreased and low levels of GNMT were expressed in the wild type mouse. GNMT was proposed to dispose of methyl groups that were not utilized by the major methyl group consumer, PEMT (318).

Epigenetic modification of DNA by methylation also requires methyl groups from SAM. DNA methylation is a critical tool in regulating tissue-specific gene expression and ultimately genome function (222). DNA methylation alters DNA protein interactions leading to either an increase or decrease in gene transcription (130). The reaction is catalyzed by a family of SAM-dependent DNA methyltransferases (DNMTs) (156), which transfer a methyl group to cytosine in CpG dinucleotides. CpG islands are usually found in gene promoter regions, thus methylation often results in regulation of DNA expression, typically repression (130). Aberrant DNA methylation is associated with a number of pathologies. Preneoplastic and tumor DNA is characterized by global hypomethylation, regional hypermethylation, and increased activity of DNMTs (20, 129, 151). Development of atherosclerotic lesions has also been associated with changes in DNA methylation (73, 110).

Transmethylation results in the production of homocysteine following the removal of adenosine from SAH. This exclusive metabolic pathway is catalyzed by *S*-adenosylhomocysteine hydrolase (SAHH) (40). This reaction is reversible favoring SAH production, and sways in the opposite direction if adenosine and homocysteine can be efficiently removed (40, 68, 217) (**Figure 2.4**).

Remethylation

Two pathways exist that function to metabolize homocysteine: remethylation, recycling methyl groups in formation of methionine, or catabolism through transsulfuration forming cystathionine (76, 153). Homocysteine can be remethylated through both folate-dependent and folate-independent mechanisms, both pathways contributing equally to the formation of methionine (85).

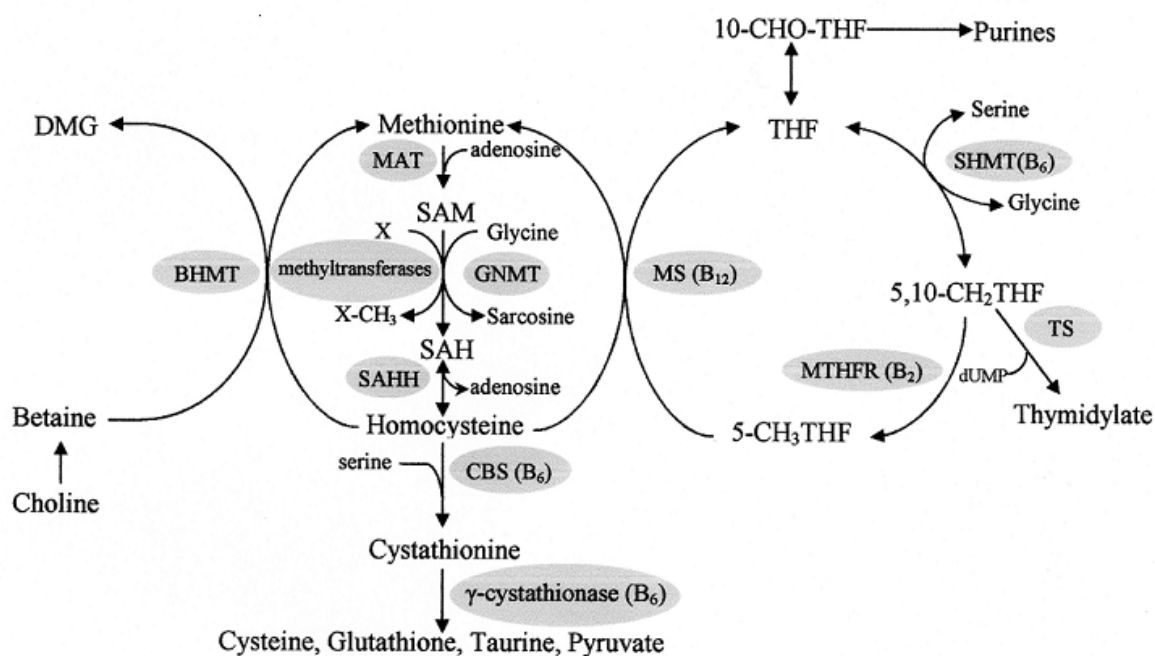


Figure 2.4. Folate, methyl group, and homocysteine metabolism

In hepatic tissue and possibly kidney, betaine derived from the oxidation of choline, serves as a source of methyl groups in the remethylation of homocysteine via the enzyme betaine homocysteine *S*-methyltransferase (BHMT) (81). This folate-independent route results in methionine and DMG (266). BHMT appears to provide a regulatory in homocysteine homeostasis, which will be discussed later in this chapter. However it is interesting to note that BHMT was induced in the CTP:phosphocholine cytidylyltransferase- α knockout mouse. This model exhibits a 20-40% increase in homocysteine concentrations because it compensates for PC production via increased PEMT activity (126). Moreover,

administration of a BHMT inhibitor in mice resulted in homocysteine concentrations that were elevated 7-fold (57).

Alternatively, remethylation occurs with the donation of a methyl group by 5-CH₃THF through the enzymatic action of MS, a vitamin B₁₂-dependent enzyme (85). Upon donation of a methyl group in the remethylation of homocysteine, 5-CH₃THF is reduced to tetrahydrofolate (THF) (289). At this point THF can either be reversibly converted to 10-CHO-THF and donate its one-carbon units in the formation of purines, or combine with serine to form 5,10-CH₂THF and glycine. This reaction is reversible catalyzed by the enzymatic activity of serine hydroxymethyltransferase (SHMT), which is dependent on vitamin B₆ as a coenzyme (235). One-carbon units from 5,10-CH₂THF, can then be donated to deoxyuridine monophosphate (dUMP) in formation of the DNA precursor thymidylate via the catalytic activity of thymidylate synthase (TS, **Figure 2.4**) (241).

The folate compound 5,10-CH₂THF, is also used in the synthesis of 5-CH₃THF, which is irreversibly catalyzed by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR), a riboflavin-dependent enzyme. At any given time the concentration of free folate compounds in the cell is insignificant leading to a struggle for folate compounds between folate binding proteins, such as MTHFR and TS (235, 264, 265). The enzyme SHMT, a B₆-dependent enzyme, provides a regulatory mechanism for distribution of one-carbon units between thymidylate and methionine synthesis that when in excess favors thymidylate. This favoring is accomplished by binding and sequestering 5-CH₃THF which in turn prevents the remethylation of methionine (107).

Transsulfuration

Catabolism of the sulfur-containing amino acid homocysteine occurs through a group of reactions known as transsulfuration. The major enzymes involved in this pathway, cystathionine β -synthase (CBS) and γ -cystathionase, are vitamin B₆-dependent. The first committed step, converting homocysteine to cystathionine is catalyzed by CBS which utilizes serine in a condensation reaction (146, 150). Finally, cystathionine is converted to the amino acid cysteine as well as other useful metabolites: glutathione, taurine, and pyruvate (262). The enzyme γ -cystathionase initiates this final step of the transsulfuration pathway (260).

Transsulfuration does not occur ubiquitously, with some cells lacking part or the entire pathway (81).

Regulation of Methyl Group Metabolism

Allosteric Regulation

In order to make certain there is an adequate supply of methyl groups for SAM-dependent transmethylation reactions a number of regulatory mechanisms exist. The enzyme GNMT is subject to allosteric regulation based on the supply of methyl groups, in the form of SAM, to optimize the SAM to SAH ratio (205). In the state of excess methyl groups through the diet or the folate-dependent one-carbon pool excess SAM binds to MTHFR, inhibiting enzyme activity (127) (**Figure 2.5**). This inhibition reduces the synthesis of 5-CH₃THF and in turn alleviates the allosteric inhibition 5-CH₃THF exerts upon GNMT resulting in the disposal of excess methyl groups. Conversely, in the state of methyl group deficiency, reduced circulating SAM concentrations results in increased activity of MTHFR, elevating concentrations of 5-CH₃THF. Further, 5-CH₃THF allosterically inhibits GNMT thereby providing methyl groups for SAM-dependent transmethylation reactions (290).

In addition to regulating methyl group supply through the enzymes MTHFR and GNMT, there are a few other points of control. Specifically, SAM is a positive allosteric regulator of CBS, the enzyme that commits homocysteine to the transsulfuration pathway (82). Excess circulating SAM concentrations will activate CBS resulting in the normalization of methyl group supply by increasing catabolism. Secondly, excess dietary protein ensuing elevated SAM concentrations will restrict remethylation enzyme BHMT, again to bring methyl group supply to an optimal state (83). However if methionine is restricted, BHMT gene expression is enhanced, conserving methyl groups (212).

Hormonal Regulation

Specific hormones have been identified as having the ability to modulate methyl group metabolism. Counter-regulatory hormones to insulin (*i.e.* glucagon and glucocorticoids) have been shown to modulate key enzymes in methyl group metabolism. In rat hepatoma cells, CBS protein abundance as well as CBS mRNA levels were increased following treatment with the synthetic glucocorticoid, triamcinolone. This effect occurred by stimulating gene expression at the transcriptional level, and the stimulatory effect was

prevented by insulin treatment (219). Moreover, treatment with the glucocorticoid dexamethasone has shown to induce GNMT activity and protein abundance in rats and rat hepatoma cells (230). Jacobs *et al.* reported that a state of hyperglucagonemia in rats induced activity of CBS, γ -cystathionase, and increased CBS mRNA levels (124). The study also revealed increased concentrations of SAM and SAH, which are allosteric activators of CBS, indicating an increase flux through the transsulfuration pathway (124). The level and synthesis rate of BHMT mRNA increased *in vitro* following triamcinolone treatment and decreased with insulin treatment (220). An earlier study also found that both triamcinolone and dexamethasone were capable of inducing MAT activity, protein level, and mRNA abundance (94). Both insulin and actinomycin D prevented this induction indicating transcriptional regulation. Interestingly both the human and rat gene for MAT reportedly contain glucocorticoid response elements within the promoter region (12, 316).

Growth hormone may also affect methyl group metabolism, namely GNMT expression. GNMT expression was induced in hypophysectomized rats, but prevented by restoring growth hormone levels with daily treatments (9). Similarly, in Ames dwarf mice which are characterized by growth hormone deficiency and an extended lifespan, GNMT activity and mRNA abundance were increased as well as MAT, BHMT, SAHH mRNA abundance. These animals also exhibit decreased hepatic SAM, increased SAH, and an overall increase in the flux of methionine through transsulfuration (35, 281, 282).

Thyroid hormone status has also been indicated in regulating folate and methyl group metabolism. MTHFR activity has been reportedly increased in hyperthyroidism and reduced in hypothyroidism, whereas the opposite was reported for MS activity (196). Accordingly, GNMT activity would be expected to decrease in hyperthyroidism due to the increased synthesis of 5-CH₃THF and subsequent allosteric inhibition (86). A study by Tanghe *et al.*

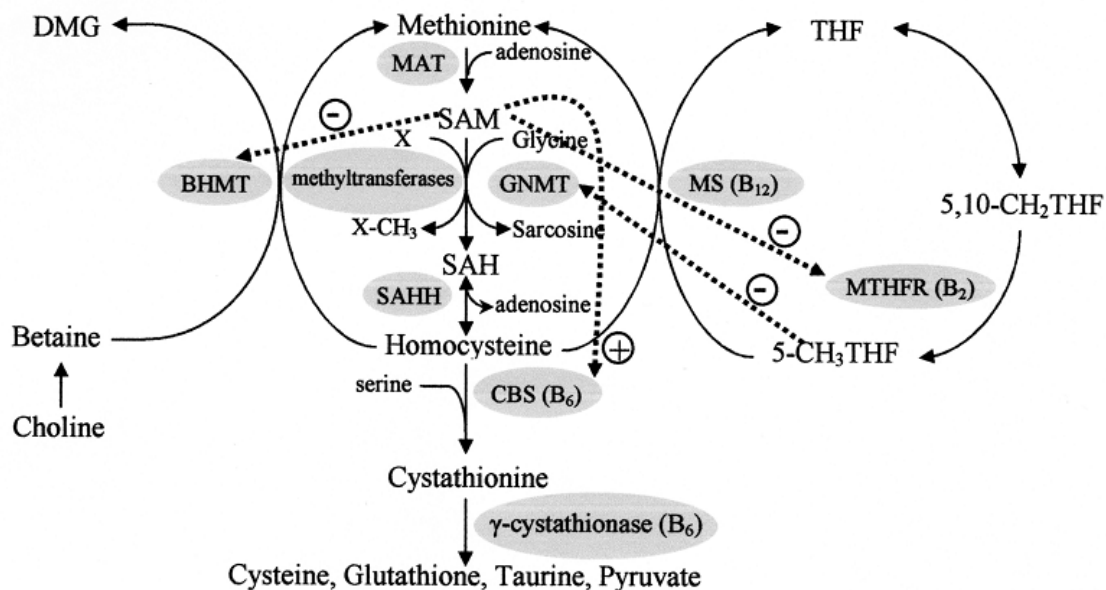


Figure 2.5. Regulation of methyl group metabolism

(269) supported this expectation when GNMT activity induced by retinoic acid was prevented by triiodothyronine treatment. Hypothyroidism has been correlated with hyperhomocysteinemia in humans (188) and significantly reduced with thyroxine therapy (207). Conversely, hypothyroidism resulted in reduced homocysteine levels in the rat which were reversed by thyroid replacement (125).

Dietary Requirements

A sufficient supply of methyl groups through the diet, from choline and/or methionine, or as a result of the folate-dependent one-carbon pool is necessary for growth and development (84). Currently, only estimates exist for methionine requirements based on nitrogen balance studies, which tend to be grouped with the other sulfur-containing amino acid, cysteine. The estimates for methionine/cysteine requirements in mg/kg/day are classified by age as follows: 3-4 months, 158; 2-5 years, 27; 10-12 years, 22; greater than 12 years, 13 (177). It is definitely necessary to fulfill protein requirements, however excessive methionine can be toxic (22). The incorporation of choline into a low-protein diet at methyl loads at the same

level or greater than methionine seems to reduce the adverse effects seen with methionine (44). Conversely, there is an adequate intake and UL stated for choline in the current DRIs. Adequate intake is given in $\mu\text{g}/\text{day}$ and classified by age as follows: 0-12 months, 125-150; 1-8 years, 200-250; 9-13 years, 375; males greater than 14 years, 550; females 14-18 years, 400; females greater than 18 years, 425. A slightly elevated choline recommendation exists for pregnant or lactating women at 450 and 500 $\mu\text{g}/\text{day}$, respectively; because large amounts are lost across the placenta and to breast milk (1, 311, 313).

Disruptions in Folate, Homocysteine, and Methyl Group Metabolism and Associated Pathologies

Folate and methyl groups are directly or indirectly involved in cell function, division, and differentiation, thus necessary for essential biological processes. Therefore insufficient supply of folate, methyl groups, or related cofactors (*e.g.* vitamin B₆ and B₁₂) can result in a multitude of pathologies as a result of disruptions in methyl group metabolism. In addition to nutrient deficiencies, treatment with retinoid compounds has been shown to perturb methyl group metabolism. Lastly, individuals that possess genetic polymorphisms in key enzymes involved in these interrelated pathways may cause alterations and susceptibility to associated pathologies.

Neural Tube Defects

Folate and methyl group metabolism are critical components in fetal development given that one-carbon units are needed in the synthesis of DNA, protein, and phospholipids (52). Neural tube defects (NTDs) are characterized by malformation of the brain and/or spinal cord in the developing embryo, instigating incomplete central nervous system development (284). NTDs are one of the most common birth defects occurring in 1 in 1000 births in the United States (3, 61).

The most common manifestations of NTDs are as congenital disorders, spina bifida and anencephaly, accounting for 50 and 40% of cases, respectively (242). Spina bifida is distinguished by incomplete closure of the spinal cord (posterior end of the neural tube) causing physical disabilities in the child. Conversely, anencephaly results in incomplete closure of the skull (anterior end of the neural tube) and is incompatible with life (98). The etiology of NTDs is continually evolving but not yet clearly understood, with over 90% of

NTD cases occurring from unknown causes (284). Identified origins of NTDs have been classified as both genetic and environmental with nutritional deficiency, specifically folate, being one of them (158-160).

Folate status has long been examined for its role in NTDs, with earlier studies reporting associations between fetal malformations and folate deficiency (109). Further interactions between NTDs and folate were found in a study that reported low folate stores in women with pregnancies affected by NTDs, in turn suggesting periconceptional folate supplementation might protect against NTDs (253). Elucidating the specific mechanism by which folate supplementation prevents NTDs is ongoing; however data currently exists supporting the beneficial role of folate supplementation in reducing the occurrence of NTDs. In particular, it was shown that folic acid treatment alone was just as effective in preventing NTDs as a multivitamin with folate (5). Moreover, a study involving 4,000 women reported 6 cases of NTDs in women receiving a placebo multi-mineral, as compared to no incidences of NTDs in the other group of women given a multivitamin containing folic acid (64). Recent reports suggest an inverse relationship between methionine intake (251) and vitamin B₁₂ status (140, 236), on the prevalence of NTDs. Periconceptional folic acid supplementation has also been shown to reduce the occurrence of non-NTD birth defects such as cleft lips, cleft palates, urinary tract, and cardiovascular congenital abnormalities, as well as limb deficiencies (63). In response to the building evidence of the effectiveness of folic acid in the prevention of NTDs, the Center for Disease Control recommended any woman of child-bearing age consume at least 400 µg of folic acid per day (6).

Hyperhomocysteinemia

Clearance of the non-protein-forming amino acid, homocysteine is dependent on remethylation and transsulfuration pathways and is possibly regulated nutritionally (244). SAM seems to be a key regulator in homocysteine metabolism since SAM allosterically inhibits MTHFR and BHMT and activates CBS. Folate as well vitamins B₆ and B₁₂ are also key factors in homocysteine metabolism. Deficiency in any of these nutrients can result in the disruption of homocysteine metabolism, and in turn hyperhomocysteinemia (99).

Homocysteine and Vascular Disease

Hyperhomocysteinemia is positively associated with vascular diseases and has been recently added to the list of commonly known risk factors for cardiovascular disease (CVD) (152, 245, 246). However the theory that homocysteine leads to vascular disease was proposed long ago when arterial lesions were discovered in children with inborn errors in methionine metabolism leading to severe hyperhomocysteinemia ($> 100\mu\text{M}$) (179, 193). Severe hyperhomocysteinemia can result from inborn errors in enzymes necessary for clearance of homocysteine, specifically CBS, MTHFR, and MS (55, 244). Normal plasma homocysteine values range from 5-15 $\mu\text{mol/L}$; whereas $\sim 10\ \mu\text{mol/L}$ is typical in men and 8 $\mu\text{mol/L}$ in women (31, 257). An elevation in plasma homocysteine concentrations by as little as 1 $\mu\text{mol/L}$ is associated with a 10% increase in the risk of CVD (24). A meta-analysis found that a 25 percent elevation in plasma homocysteine ($\sim 3\ \mu\text{mol/L}$) was associated with a 10 percent increase risk for developing cardiovascular disease and a 20 percent greater risk of stroke (4). A prospective study found a 27 percent elevated risk of venous thromboembolism with a 5 $\mu\text{mol/L}$ increase in plasma homocysteine (71). Folate deficiency has also been linked to elevated plasma homocysteine, with a 9.8-fold increase reported in folate-deficient rats as compared to folate-replete controls (185). A reduction in plasma homocysteine concentrations were also evident following administration of folic acid promoting remethylation (33, 171, 210, 300). Therefore it is estimated that increasing folate intake by 200 μg per day will result in a reduction in homocysteine by 4 $\mu\text{mol/L}$ (31, 285).

Research is ongoing as to whether homocysteine actually imposes atherogenic effects or if it just an indicator of another mechanism (279). Many studies do support the hypothesis that homocysteine is directly involved in vascular disease by causing degeneration of the vascular endothelium (158). Particularly, a study using cultured endothelial cells has demonstrated cytotoxic effects following homocysteine treatment (294). In addition an *in vivo* study revealed the rapid formation of plaques following injection of homocysteine thiolactone (180).

Studies in apolipoprotein E (apoE) and CBS knockout mice have revealed additional insight into the role of homocysteine in vascular disease. ApoE is involved in the removal of lipoproteins from the plasma via low density lipoprotein receptors. ApoE-null mice are

severely hypercholesterolemic and develop spontaneous aortic atherosclerotic lesions (214, 317). The deletion of the CBS gene creates a model of hyperhomocysteinemia (297). Hyperhomocysteinemia has been shown to enhance the formation of lesions leading to arterial injury in apoE knockout mice (112). A study using double knockout mice led to aortic lesions regardless of dietary manipulation. Moreover uptake of modified low density lipoproteins by macrophages was increased with elevated homocysteine concentrations (295). Monocyte-derived macrophages and smooth muscle cells that accumulate low-density lipoproteins are called foam cells and have been shown to contribute to atherosclerotic lesions (104). Homocysteine has also been shown to increase foam cells and oxidized low-density lipoprotein content, as well as lead to the accumulation of cholesterol and hydrogen peroxide in cultured monocytes (128). Superoxide dismutase, the free radical scavenger enzyme, was also reduced by homocysteine treatment and it was suggested that this effect was mediated by methylation. Oxidative radicals have also been implied as contributors to endothelial dysfunction in animals (47, 66, 67, 75, 280, 298). Studies indicated that elevated homocysteine levels results in endothelial cell dysfunction in humans (306) as well as vascular smooth muscle cell proliferation (166). Additional mechanisms indicate homocysteine may induce vascular disease by causing platelet function abnormalities; however the exact mechanism is yet to be revealed.

Homocysteine and Neural Tube Defects

Elevated homocysteine is consistently associated with NTDs which again may be as a result of inborn errors in the enzymes (MTHFR and MS) involved in remethylation of homocysteine (190, 283). Further an association has been identified between NTD births and increased cellular folate depletion. This was thought to be a result of impaired folate remethylation to THF the main intracellular form of folate (161). This further identifies the need to promote periconceptional vitamin supplementation, specifically folate.

Cancer

As explained previously, folate is directly and indirectly involved in the modification and synthesis of DNA. Therefore, folate deficiency has been identified as a factor contributing to cancer development and enhancement (170). Mechanisms for this relationship have been proposed but not yet clearly defined (16). One hypothesis of this relationship is in the

donation of one-carbon units from 5,10-CH₂THF in the synthesis of the DNA precursor thymidylate. Inadequate folate can lead to misincorporation of uracil into DNA due to lack of thymidylate. This misincorporation can lead to stress of the DNA repair mechanism and in turn cause DNA strand breaks and chromosome instability. However misincorporation of uracil and subsequent DNA damage seems to be reduced by folate supplementation (25).

Another proposed mechanism for this relationship between folate and cancer is the role of folate in DNA methylation. DNA methylation has shown to regulate gene expression, with lack of methylation typically leading to gene expression, and increased methylation resulting in gene suppression (289). In addition to methyl groups being provided by protein in the diet, folate indirectly plays a role in methylation of DNA by donating a methyl group to homocysteine in the generation of SAM. Therefore, it has been observed that rats fed a diet lacking folate, methionine, choline, and vitamin B₁₂, display decreased SAM concentrations and hepatic DNA methylation (293). Global DNA hypomethylation has shown to precede cancer development, specifically due to hypomethylation of proto-oncogenes which increases transcription of associated mRNAs (72).

An additional hypothesis for the role of folate in cancer is in cytotoxic natural killer cells. Natural killer cells provide important immune protection against tumor cells (274). It was proposed that in folate deficiency, these cells have reduced ability to destroy cancerous cells (170). Further research suggests that natural killer cell-mediated cytotoxicity is dependent on the state of folate deficiency with severe folate deficiency in rats resulting in the reduced ability for cytotoxicity. However moderate folate deficiency, associated with increased risk of some cancers, did not affect cytotoxicity capabilities of natural killer cells (139).

Accumulating evidence suggests that folate may play a role in development and progression of many cancers including: colorectum, lungs, pancreas, esophagus, stomach, cervix, breast, and leukemia (136). The most evidence indicating a relationship between folate and cancer development has been documented in colorectal cancer (138, 218). Several retrospective studies have suggested a decreased risk of colorectal neoplasms in subjects with the highest folate intake; whereas folate deficiency increases the risk of colorectal cancer (18, 95). However folate may have a dual relationship in colorectal

carcinogenesis. Folate deficiency may prevent the progression of established colorectal neoplasms, but promote neoplastic formation in normal mucosa (137).

Megaloblastic Anemia

Disturbed DNA synthesis as a result of folate and/or B₁₂ deficiency can result in the megaloblastic anemia. This form of anemia manifests in large, immature, abnormal, red blood cell precursors in the bone marrow and peripheral blood (133). The role folate and vitamin B₁₂ play in this pathology is in the pathway converting 5-CH₃THF to THF through the activity of MS (233). In the case that there is vitamin B₁₂ deficiency, MS activity is reduced essentially trapping methyl groups in the form 5-CH₃THF. This “methyl trap” decreases the availability of folate coenzymes for purine and pyrimidine biosynthesis, which are needed for red blood cell division and differentiation (248).

This somewhat pseudo folate deficiency that occurs can result in a reduction of SAM and in turn methyl groups for transmethylation reactions including myelin basic protein. A decrease in methyl groups for synthesis of myelin basic protein can cause demyelination and in turn a state of neuropathy (239). Therefore, it is crucial that individuals suffering from megaloblastic anemia not only supplement folate, but vitamin B₁₂ to prevent associated neuropathy. However, steps should be taken to discover the root of the problem because supplementing folate can mask vitamin B₁₂ deficiency and exacerbate associated neurological problems (30).

Neurological/Neuropsychiatric Disorders

Neurological disorders associated with folate and/or B₁₂ deficiency are not quite as well understood as the hematological disorders. Vitamin B₁₂ and folate deficiencies have been associated with neurological and neuropsychiatric disorders (29). A hypothesis for the role of these nutrients is again through the reaction catalyzed by MS. MS, which utilizes both vitamin B₁₂ and folate, in the formation of methionine and in turn SAM. SAM provides methyl groups in the synthesis of proteins, phospholipids and neurotransmitters such as serotonin, norepinephrine, and dopamine. Specifically reduced serotonin levels and activity have been reported in the brain of folate-deficient rats and humans (28, 119). It is interesting to note that 5-CH₃THF is transported across the blood brain barrier, and the concentration in

the cerebrospinal fluid is three times greater than in serum (30). This further indicates the importance of the MS-catalyzed pathway.

Several studies have reported a high incidence of folate deficiency accompanying depression (43, 189). Studies have also been conducted to determine the efficacy of folate and SAM supplementation in these affective disorders, whereas supplementation seemingly promotes antidepressant activity (89, 97). However it is not clear if folate deficiency actually plays a role in the development of depressive illnesses or if it is secondary to poor appetite.

Recent studies have also linked the rise in homocysteine, resulting from folate deficiency and reduced enzyme activity, to two major neurological disorders: Alzheimer's disease and Parkinson's disease (178). Typically serum homocysteine levels are elevated in folate- or vitamin B₁₂-deficient patients (11). There are several suspected mechanisms for the role of folate and homocysteine in neuronal damage. One of these mechanisms is folate deficiency is thought to induce DNA damage by reducing homocysteine remethylation to SAM for transmethylation reactions (246). Another possible mechanism is the accumulation of SAH. Elevated homocysteine creates a favorable condition for the hydrolysis of homocysteine to SAH by SAHH. SAH accumulation will inhibit SAM-dependent transmethylation reactions, and in turn induce DNA damage and cell apoptosis (148). In addition, it has been shown that neurons cultured with homocysteine deplete their ATP reserves in attempts to repair damaged DNA. ATP depletion is thought to be a key factor in the degeneration of neurons in Alzheimer's and Parkinson's diseases (148).

Genetic Profile

Genetic polymorphisms are among the factors that can perturb folate, homocysteine, and methyl group metabolism resulting in associated pathologies. These genetic differences are in key enzymes in one-carbon metabolism and typically result in reduced activity. Several of these polymorphisms have been identified as well as the possible side effects of their existence.

MTHFR Polymorphism

The enzyme MTHFR catalyzes the irreversible conversion of 5,10-CH₂THF to 5-CH₃THF, which donates its methyl group in the remethylation of homocysteine. The most common mutation of this enzyme is autosomal recessive, occurring at base pair 677, and

resulting in a C→T substitution (C677T) causing valine to substitute for alanine in the enzyme (90). The frequency of this mutation varies depending on race and ethnic background, but it is estimated to occur at rate of 12% in Caucasian and Asian populations homozygous for the trait, and up to 50% for those who are heterozygous for the trait (33).

Individuals that are homozygous for this mutation exhibit reduced activity and stability of MTHFR. Studies indicate that persons with the homozygous C677T mutation have reduced plasma folate concentrations and elevated plasma homocysteine concentrations (33). Modest elevations in homocysteine is an independent risk factor for vascular disease and NTDs; however studies are conflicting as to whether the homozygous C677T mutation is itself a risk factor in vascular disease and NTDs (17). The relationship between cancer and the homozygous C677T mutation seems to be dependent on folate status. A decreased risk for colorectal cancer was found in men with normal plasma folate concentrations and the MTHFR mutation by increasing the availability of one-carbon units for nucleotide synthesis. Conversely, the men with low folate status seemed to counteract the protective effect of the mutation (165).

MS Polymorphism

The enzyme MS has also been identified in the list of mutations associated with one-carbon metabolism. The most common MS polymorphism is located at base pair 2756 causing an A→G (A2756G) substitution and resulting in glycine replacing aspartic acid in the functional enzyme (48). It is not yet clear whether this mutation changes activity or abundance of the enzyme; however most research indicates there is no association between this mutation and homocysteine concentrations (120, 141). Most studies also indicate that there is no association between the homozygous mutation for MS and the risk for vascular disease (164, 187, 276). Although a study by Kerk *et al.* (141) did find that subjects homozygous for the MS mutation exhibited a four-fold increase in the risk of coronary heart disease as compared to individuals with the wild-type or heterozygous genotypes.

CBS enzyme deficiency

The transsulfuration pathway beginning, with the CBS activity, plays a crucial part in the removal of homocysteine when the supply of methyl groups is in excess (85). Therefore, a deficiency in CBS activity results in hyperhomocysteinemia, homocystinuria, methionine in

the plasma and urine, decreased cystathionine and cysteine levels, in addition to the possible manifestation of a number of pathologies (145, 244). Specifically, osteoporosis, scoliosis, arteriosclerosis, thromboembolism, convulsions, and psychiatric disturbances are some pathologies that can develop from this enzyme deficiency (142), and as many as 50% of those who go untreated, develop mental retardation (308). The exact mechanism by which CBS enzyme deficiency is associated with diseases is not yet known.

CBS enzyme deficiency is an autosomal recessive trait and the most common inborn error of methionine metabolism occurring in approximately 1 in 200,000 individuals worldwide (246). Greater than 60 mutations have been identified in the CBS gene (144). The most severe mutations occur at base pair 833 resulting in a T→C (T833C) substitution and at base pair 919 resulting in a G→A substitution (G919A). These mutations are fairly rare affecting less than 1% of the general population (143). The most common mutation is caused by a 68 base pair insertion between nucleotides 844 and 845 (844ins68). This mutation occurs in the heterozygous form in approximately 12 % of the U.S. population (275).

CBS is a vitamin B₆-dependent enzyme, therefore some patients with CBS deficiency respond to vitamin B₆ supplementation resulting in lower homocysteine concentrations. It is estimated that approximately 50% of those with CBS enzyme deficiency respond to high doses of vitamin B₆, evident by lowered homocysteine concentrations (195). However, many individuals do not respond to vitamin B₆ supplementation and therefore must rely on a methionine-restricted diet. Vitamins B₁₂, B₆, and folate may further reduce homocysteine concentration in these resistant individuals (301). Betaine supplementation has also been tested in mouse models of CBS deficiency and humans and has been found to consistently lower plasma homocysteine concentrations. The only concern is the elevated methionine levels that result, however most patients seem to remain below 1500 µmol/L, a level that has not been associated with adverse effects (238, 301, 302).

GNMT polymorphism

Reports of mutations in the gene that codes for GNMT have been reported in humans (192), and has since been mimicked in a GNMT knockout mouse model (162). The mutations found in siblings with severe enzyme deficiency were two single nucleotide

substitutions: (i) T→C at nucleotide 1481, and (ii) C→A at nucleotide 3715. The transitions ultimately changed leucine to proline and histidine to asparagine, respectively, in the translated protein (13, 163). These patients exhibited severe hypermethioninemia and normal plasma sarcosine levels despite a drastic elevation in plasma SAM levels, as well as elevated liver transaminases and hepatomegaly.

An additional study examining GNMT in human liver cancer progression identified six novel polymorphisms (277), since GNMT is typically diminished in hepatoma cells (14, 49, 102). Further examination of the 1289 C→T (C1289T) polymorphism (TT genotype), which was associated with cancer risk (277), found higher homocysteine concentrations in women following folate restriction as compared to CT or CC genotypes (21). This effect was exacerbated by the presence of the MTHFR C677T mutation.

Retinoids

Factors both nutritionally and hormonally have been identified as having the ability to alter methyl group metabolism by targeting key enzymes involved. Specifically the vitamin A derivatives, all-*trans*-retinoic acid (RA), and 13-*cis*-retinoic acid (CRA) are among these factors. More importantly these alterations may affect those who utilize these derivatives therapeutically. CRA (Isotretinoin/Accutane®) is used in dermatology and taken orally to treat cystic acne that is unresponsive to topical treatment; whereas RA (Vesanoid®) is used in oncology to treat promyelocytic leukemia (206, 213).

Retinoid administration has been shown to affect one-carbon metabolism by increasing methionine catabolism as well as inducing hypomethylation of DNA by activating GNMT in rats (77, 229, 234). An additional study also found elevated homocysteine concentrations in humans treated with isotretinoin, linking this finding to either CBS inhibition or liver dysfunction (237). CRA and RA have been shown to induce GNMT activity and protein abundance *in vivo* (182, 208, 231) as well as induction of GNMT by RA *in vitro* (230). Induction of GNMT results in a loss of methyl groups for subsequent SAM-dependent transmethylation reactions. This effect seems to be dose-dependent with as little as 5 µmol/kg body weight and a single dose causing significant induction and a maximal effect at 30 µmol/kg body weight and four days of treatment (208). Activation of GNMT by retinoids also exhibited tissue and gender specificity. McMullen *et al.* reported male rats were more

sensitive to retinoid-mediated activation of GNMT than female rats (182). Retinoid administration resulted in elevated hepatic GNMT protein and abundance, but did not affect renal or pancreatic GNMT (182).

RA is known to induce a gluconeogenic state which in turn alters methyl group metabolism through the induction of key gluconeogenic enzymes including phosphoenolpyruvate carboxykinase, the rate-limiting step of gluconeogenesis (209, 250). Interestingly, GNMT is located primarily in gluconeogenic tissues implying a possible role in hormonal regulation. These modifications of GNMT activity by RA are thought to occur transcriptionally, however there is currently no report of a retinoic acid response element on the promoter region of GNMT (209). Phosphorylation of GNMT has been shown to modulate activity of the enzyme. Wagner *et al.* found *in vitro* that phosphorylation, by cAMP-dependent protein kinase, of GNMT resulted in a 2-fold increase in the activity of the enzyme; however if 5-CH₃THF was first bound to GNMT, phosphorylation was inhibited as well as enzyme activity (292).

Diabetes Mellitus

Diabetes-Perturbed Folate, Homocysteine, and Methyl Group Metabolism

A diabetic state is commonly characterized by chronic hyperglycemia and defects in the action and/or secretion of insulin. Diabetes leads to disturbances in macronutrient metabolism and ultimately damage and dysfunction of tissues especially in the eyes, kidneys, and vascular system (23). A diabetic state has also been indicated in directly altering enzymes involved in folate, homocysteine, and methyl group metabolism. In a study by Xue and Snoswell, a 65-fold induction in GNMT activity was reported in alloxan-induced diabetic sheep (307). Similarly, in an alloxan-induced diabetic state or a starvation state in rats, resulted in a 2-fold induction in GNMT activity (310). Increased GNMT activity and abundance, CBS abundance, and BHMT activity, as well as decreased homocysteine and MS activity have also been shown in streptozotocin-induced diabetic rats (198).

Modifications in homocysteine concentrations are also evident in a diabetic state, but appear to be dependent on the status of the kidneys (27, 305). The kidney is considered the main site of homocysteine uptake and metabolism (26), and renal hyperfiltration is characteristic of early renal dysfunction in diabetes (272). If renal function is sufficient, a

diabetic condition results in a marked decrease in plasma homocysteine concentrations. However, if diabetes is coupled with renal dysfunction, the outcome is elevated plasma homocysteine levels (108, 216). Elevated homocysteine is an independent risk factor for vascular disease, and is considered to be the major cause of death in diabetics (223). In a study by Jacobs *et al.* (123), researchers examined this effect in streptozotocin-induced diabetic rats and found plasma homocysteine concentrations were reduced by 40%; however treatment with insulin prevented the decrease in homocysteine, indicating insulin may be involved in the regulation. This decline in homocysteine likely manifests through the modification of transsulfuration enzymes, cystathionine β -synthase and γ -cystathionase (123). Further, in streptozotocin-induced diabetic rats elevated CBS mRNA abundance was prevented by insulin administration (219). Homocysteine kinetic studies in healthy humans revealed increased homocysteine clearance following insulin infusion (271). Folate-independent remethylation may also contribute to reduced plasma homocysteine concentrations since activity and mRNA of BHMT were elevated in streptozotocin-induced diabetic rats (197, 198, 220).

Similar disruptions in methyl group metabolism have been exhibited in a model of type 2 diabetes, the leptin-receptor defective (db/db) Zucker diabetic fatty (ZDF) rat. Total plasma homocysteine levels were reduced in these animals at both the pre-diabetic insulin resistant stage (5 weeks) and early type 2 diabetes (11 weeks) (299). Renal hyperfiltration and proteinuria have been reported in ZDF rats up to three months of age. Filtration rate typically decreases, to the level reported in normal rats, but proteinuria has been shown to increase further by seven months of age (115). Similarly, lower serum homocysteine concentrations have also been reported in non-diabetic insulin-resistant patients (226). Flux studies in type 2 diabetic patients with nephropathy reveal hyperhomocysteinemia regardless of insulin stimulation; owing to decreased homocysteine clearance and transsulfuration (271).

Reduced plasma homocysteine concentrations in ZDF rats are likely due to increased clearance of homocysteine via increased activity of BHMT, γ -cystathionase, and CBS (299). The increased BHMT activity was accompanied by a depletion of hepatic betaine concentrations in the rats as well as a reduction in the export of homocysteine from

hepatocytes isolated from the ZDF rats (299). Due to increased folate-independent remethylation, diabetics may have an increased betaine requirement. In support of this hypothesis, betaine supplementation was found to reduce homocysteine concentrations in healthy human subjects as well as MTHFR-deficient (258) and CBS-deficient mice (238). Hepatic SAM and SAH were also increased in ZDF rats, but an overall decrease in the ratio of SAM to SAH was reported. Decreased lymphocytic MTHFR activity, increased erythrocyte SAH, and decreased erythrocyte SAM and the ratio of SAM:SAH have been shown with the progression of nephropathy in diabetic patients (216). An additional study in type 2 diabetic patients found increased plasma homocysteine, SAM, and SAH, but a decrease in the plasma SAM:SAH ratio with the progression of renal insufficiency (108). Decreased SAM and the SAM:SAH ratio may indicate a mechanism for hyperhomocysteinemia in diabetic nephropathy since SAM stimulates CBS activity, the rate-limiting step of homocysteine catabolism (79, 261).

Vitamin D and Diabetic Pathogenesis

Vitamin D is obtained through limited sources in the diet such as fish, eggs, meat, and fortified foods and endogenously synthesized in the skin from a cholesterol derivative following sun exposure (173). The major circulating form of vitamin D, 25-hydroxyvitamin D₃ (*i.e.* 25(OH)D₃, cholecalciferol, or vitamin D₃), requires two hydroxylation reactions to form the active metabolite 1,25-dihydroxyvitamin D₃ (*i.e.* 1,25(OH)₂D₃ or calcitriol). The hydroxylation reactions are catalyzed by enzymes in the P450 family, the first reaction occurring in the liver by 25-hydroxylase (173). The second hydroxylation reaction occurs most commonly in the kidney by 1 α -hydroxylase but is also expressed in dendritic, macrophage, colon, placenta, breast, prostate, and pancreas cells (167). Vitamin D metabolites circulate attached to a chaperone protein called vitamin D binding protein. At the site of interest 1,25(OH)₂D₃ functions metabolically by binding to the nuclear vitamin D receptor, altering the transcription of genes regulated by 1,25(OH)₂D₃ (173). Vitamin D classically functions in the metabolism and maintenance of calcium and bone.

Since receptors for the metabolically active form of vitamin D have been identified in tissues unrelated to calcium and bone metabolism, particularly the β -cells of the pancreas, the classical role of vitamin D is expanding. A growing collection of evidence indicates a

relationship between vitamin D and diabetes. In non-obese diabetes-prone (NOD) mice, treatment with $1,25(\text{OH})_2\text{D}_3$, prevented the onset of type 1 diabetes (175, 315) and has also been shown to improve streptozotocin-induced diabetes (70, 174). This effect was not reported in vitamin D receptor knockout mice (174). Moreover vitamin D deficiency seemingly accelerates diabetes onset in NOD mice (96, 315). It has also been suggested that increasing vitamin D intake as early as infancy, may decrease the risk of the child developing type 1 diabetes later in life (7, 121, 259).

In type 2 diabetic humans and animals, vitamin D deficiency has been reported to inhibit insulin secretion and impair glucose tolerance (54, 93, 202). Further, vitamin D repletion seems to ameliorate the noted abnormalities in insulin secretion and glucose tolerance (131, 203). Glucose concentrations in type 2 diabetic rats were reduced by 40% with vitamin D_3 supplementation (69). Reduced circulating insulin and elevated blood glucose have been reported in mice without a functional vitamin D receptor (314). Epidemiological evidence has also indicated that serum 25-hydroxyvitamin D_3 levels in adults are inversely correlated with the occurrence of type 2 diabetes, insulin resistance (243), and metabolic syndrome (87, 167). However these associations have yet to be confirmed by intervention studies.

Summary

Folate, homocysteine, and methyl group metabolism function in concert to mobilize and activate one-carbon units, primarily methyl groups, in the biosynthesis and modification of essential compounds necessary for optimal health. Thus, disruption of these critical pathways has been associated with a number of pathologies including cancer, vascular disease, and birth defects. A number of factors have been identified as having the ability to disrupt folate, homocysteine, and methyl group metabolism. Specifically, we and others have reported that a diabetic state disrupts these metabolic pathways, possibly connecting diabetes to complications of the disease. The research described in the following chapters was aimed at preventing disruptions in folate, homocysteine, and methyl group metabolism resulting from a diabetic state.

CHAPTER 3: MODULATION OF METHYL GROUP BY INSULIN TREATMENT IN DIABETIC RATS

Modified from a paper published in the *Journal of Nutrition*¹ and an abstract published in the *Federation of American Societies for Experimental Biology Journal*²

Abstract

Modifications in methyl group and homocysteine metabolism are associated with a number of pathologies including vascular disease, cancer, and neural tube defects. A diabetic state is known to alter both methyl group and homocysteine metabolism depending on renal function. Elevated plasma homocysteine results in a diabetic state with compromised renal function, whereas diabetes without renal dysfunction is characterized by hypohomocysteinemia. Glycine *N*-methyltransferase (GNMT) is a major regulatory protein that controls the supply and utilization of methyl groups. We have shown previously that a diabetic state: (i) induces GNMT activity as well as protein and mRNA abundance, and (ii) reduces plasma homocysteine pools by stimulating its catabolism and folate-independent remethylation. This study was conducted to determine if insulin plays a direct role in the regulation of homocysteine concentrations and GNMT. Rats were randomly assigned to one of three groups: control, streptozotocin (STZ)-induced diabetic (60 mg/kg BW), and insulin-treated diabetic (1.0 unit, *b.i.d.*). After 5 days, the 1.5-fold elevation in GNMT activity and hypohomocysteinemia in diabetic rats was completely prevented by insulin treatment. Additionally, disruptions in methionine synthase (MS), phosphatidylethanolamine *N*-methyltransferase (PEMT), and DNA methylation were also prevented by insulin administration. Similar findings have been reported in a type 2 diabetic model, thus we hypothesized that glucose or insulin levels may represent a regulatory signal to modify GNMT and homocysteine. In support of this hypothesis, blood glucose concentrations were negatively correlated with total plasma homocysteine ($r = -0.49$, $p = 0.05$), and positively correlated with GNMT activity ($r = 0.80$, $p = 0.0001$). Future research will focus on elucidating the role of glucose or insulin as a signal for regulating homocysteine and methyl group metabolism.

Introduction

The primary methyl donor, *S*-adenosylmethionine (SAM), requires a constant supply of methyl groups for numerous transmethylation reactions including the biosynthesis and modification of nucleic acids, proteins, and phospholipids ($X \rightarrow X\text{-CH}_3$, **Figure 3.1**) (176, 289). Methionine, provided through the diet or endogenously via homocysteine remethylation, is activated by methionine adenosyltransferase (MAT) forming SAM (176, 194). SAM donates methyl groups in over 50 known mammalian transmethylation reactions, including the synthesis of phospholipids, namely phosphatidylcholine (PC) (289). PC is synthesized from phosphatidylethanolamine (PE) through the activity of PEMT. PEMT is a primary consumer of methyl groups from SAM and thought to be a major contributor to homocysteine production (126, 256).

Epigenetic modification of DNA by methylation also requires methyl groups from SAM. DNA methylation is a critical tool in regulating tissue-specific gene expression and ultimately genome function (222). The reaction is catalyzed by a family of SAM-dependent DNA methyltransferases (DNMTs) (156), which transfer a methyl group to cytosine in CpG dinucleotides. Aberrant DNA methylation is associated with a number of pathologies, including carcinogenesis and atherosclerosis (20, 73, 110, 129, 151).

Management of methyl group supply is essential and accomplished through the enzymatic activity of GNMT. GNMT is an abundant hepatic cytosolic protein, comprising approximately one to three percent of all soluble cytosolic protein (135, 205). GNMT functions in optimizing the SAM:*S*-adenosylhomocysteine (SAH) ratio using methyl groups from SAM to convert glycine to sarcosine and SAH (310). The SAM:SAH ratio is an indicator of transmethylation potential since SAH is a potent inhibitor of most transmethylation reactions (40, 135), except GNMT which is only weakly inhibited (268). Alternatively, GNMT activity is allosterically regulated by the folate coenzyme 5-methyltetrahydrofolate (5-CH₃THF) in response to methyl group supply (309). SAM is a negative allosteric regulator for 5,10-methylenetetrahydrofolate reductase (MTHFR), the enzyme responsible for 5-CH₃THF production (127, 149). Excess methyl groups in the form of SAM inhibit MTHFR activity and 5-CH₃THF synthesis. This alleviates the negative allosteric regulation of GNMT by 5-CH₃THF, and allows disposal of excess methyl groups in

the conversion of glycine to sarcosine. Alternatively, during conditions of methyl groups shortage, the negative regulation of MTHFR by SAM is alleviated. As a result, synthesis of 5-CH₃THF is increased, inhibiting GNMT activity and conserving methyl groups for transmethylation reactions (291). A lack of methyl groups for these essential reactions may also result in a number of pathologies including carcinogenesis, neural tube defects, and cardiovascular disease (132, 227, 240). The non-protein forming amino acid homocysteine is produced following the hydrolysis of SAH by SAH hydrolase (SAHH)(38, 51). Homocysteine is either remethylated to methionine or catabolized through the transsulfuration pathway to homocysteine.

The irreversible catabolism of homocysteine ultimately results in cysteine via the transsulfuration pathway. Transsulfuration is the result of two B₆-dependent enzymes. Cystathionine β -synthase (CBS) catalyzes the first reaction, the condensation of homocysteine with serine, resulting in cystathionine production. Finally, cysteine is produced in a reaction catalyzed by γ -cystathionase (147). Homocysteine remethylation occurs by both folate-dependent and folate-independent mechanisms, which have been found to contribute equally to the remethylation of homocysteine (85). Folate-dependent remethylation requires donation of a methyl group from 5-CH₃THF. This reaction is catalyzed by the B₁₂-dependent enzyme MS, resulting in methionine and tetrahydrofolate (THF) (248). Betaine, derived from the oxidation of choline, is the methyl group donor in folate-independent remethylation. Betaine-homocysteine S-methyltransferase (BHMT) catalyzed this reaction, forming dimethylglycine and methionine (91).

Several factors have been identified as having the ability to influence homocysteine and methyl group metabolism by disrupting the expression of key enzymes involved. A diabetic state, characterized by reduced insulin levels, elevated glucose, and elevated circulating counter-regulatory hormones (*i.e.* glucagon and glucocorticoids) (304), has been identified in the disruption of homocysteine and methyl group metabolism. Altered homocysteine concentrations are commonly reported and have been shown to be inversely correlated with kidney function (232, 305). In both type 1 and 2 diabetes without renal complications hypohomocysteinemia has been found in humans and rats (8, 123, 198, 224, 299). Similar

findings have also been reported in animals and cells treated with glucocorticoids and glucagon (124, 219, 230).

Hypohomocysteinemia likely results from increased transsulfuration as evidenced by increased CBS activity, protein, and mRNA levels as well as γ -cystathionase activity (198, 219, 299). Folate-independent remethylation may also assist in the reduction homocysteine via BHMT; its activity and mRNA levels have been reportedly increased in acute diabetes (198, 220, 299). However, as renal function declines in chronic diabetes, homocysteine levels rise (53, 118, 216). As previously noted, elevated plasma homocysteine is currently considered an independent risk factor for the development of vascular disease (31, 55, 132).

We have shown a diabetic state induced by streptozotocin (STZ) leads to the disruption of these vital pathways. This disruption is characterized by induction of GNMT and PEMT, a reduction in methionine synthase activity, as well as altered homocysteine metabolism (197, 198). Typically insulin is administered in a type 1 diabetic state, to control blood glucose levels. Consequently, this research was conducted to determine if insulin (INS) prevents STZ-induced diabetic perturbations in methyl group metabolism.

Materials & Methods

Chemicals

Reagents used in this study were obtained from the following sources: *S*-adenosyl-L-[*methyl*- ^3H]methionine, PerkinElmer Life Sciences; chemiluminescent Western blotting detection reagents, GE Healthcare; streptozotocin, *S*-adenosyl-L-methionine, and porcine insulin, Sigma-Aldrich; goat anti-mouse IgG horseradish peroxidase, Southern Biotechnology; HpaII endonuclease and BssHII endonuclease, New England Biolabs Inc.; [^3H]-dCTP, NEN Life Science Products. The GNMT antibody was generously provided by Dr. Yi-Ming Chen of the National Yang-Ming University, Taipei, Taiwan (155). All other chemicals were of analytical grade.

Animals

Male Sprague-Dawley rats (125-149 grams) were kept in individual plastic cages and housed in a room with a 12-hour light:dark cycle. Animals were allowed access to food and water *ad libitum*. Following a 6 day acclimation period on the control diet (230) each animal was randomly assigned to one of three treatment groups: control, STZ-induced diabetic

(STZ), or insulin (INS)-treated diabetic (STZ+INS). At the end of the acclimation period animals received a single intraperitoneal injection of STZ (60 mg/kg body weight) or the vehicle (10 mM citrate buffer, pH 4.5). The following day rats were given insulin injections (1 unit/200 μ L saline), *b.i.d.*, every 12 hours (88), for a period of 5 days or the vehicle alone. Animals were anesthetized the next day with a mixture of ketamine:xylazine (90:10 mg/kg body weight) and heparinized whole blood was collected by cardiac puncture. An aliquot was removed for the determination of blood glucose concentrations using a commercial kit (Sigma-Aldrich). The remaining whole blood was centrifuged at $5,000 \times g$ for 5 minutes and the plasma was removed and stored at -20°C for subsequent determination of homocysteine concentrations. Immediately following blood collection, portions of the liver were removed and homogenized in ice-cold phosphate-buffered (10 mM, pH 7.0) sucrose (0.25 M) containing 1 mM EDTA, 1 mM sodium azide, and 0.1 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at $20,000 \times g$ for 30 minutes and an aliquot of the supernatant was stored at -70°C for determination of GNMT activity and abundance, and MS activity. An additional aliquot was removed and centrifuged at $100,000 \times g$. The pellet was collected for analysis of PEMT activity by resuspending the pellet in 0.25 M sucrose buffer and storing at -70°C . Additional portions of the liver were collected and snap-frozen in liquid nitrogen for methylation analysis and quantification of hepatic SAM and SAH concentrations. Total soluble protein concentration of the cytosolic and microsomal fractions were determined using a commercial kit (Coomassie Plus, Pierce) based on the Bradford method (32) and bovine serum albumin as the standard.

GNMT Activity Analysis

The enzymatic activity of GNMT was determined using the method described by Cook and Wagner (59) with minor modifications. The assay mixture (100 μ L) contained 0.2 M Tris buffer (pH 9.0), 2 mM glycine, 5 mM dithiothreitol, 1 mM S-adenosyl-L-[*methyl*- ^3H]methionine, and was initiated with 250 μ g protein followed by incubation at 25°C for 30 minutes. Trichloroacetic acid (10%, w/v) was added to terminate the reaction followed by the addition of activated charcoal and centrifugation ($14,000 \times g$) to remove radiolabeled SAM. Aliquots of the supernatants were removed for liquid scintillation counting.

Determination of GNMT Abundance

GNMT protein abundance was quantified using immunoblotting techniques as described by Rowling *et al.* (230). A 10-20 percent (w/v) gradient SDS-polyacrylamide gel was used to quantify the 32-kDa monomer subunit of GNMT. Following separation, proteins were transferred to a nitrocellulose membrane and incubated overnight at 4°C with a 1:5,000 dilution of the monoclonal GNMT antibody (155). The membrane was then incubated with goat anti-mouse IgG horseradish peroxidase secondary antibody for 1 hour at room temperature. GNMT protein abundance was visualized with chemiluminescence and exposed to Kodak X-Omat AR film. Densitometric analysis was performed using SigmaGel software (SPSS).

Total Plasma Homocysteine Analysis

Total plasma homocysteine concentrations were determined using HPLC and fluorescence detection (278). Plasma, derivatized with 10 percent (v/v) tributylphosphine in dimethylformamide, was incubated at 4°C for 30 minutes. The reaction was terminated with ice-cold trichloroacetic acid containing 1 mM EDTA. Following centrifugation at $1,000 \times g$ for 5 minutes, supernatants were added to a solution containing 0.125 M borate buffer (pH 9.5), 1.55 M sodium hydroxide, and 0.1 percent (w/v) 4-fluoro-7-sulfobenzofurazan (ammonium salt). N-acetylcysteine (1 mM) was also added to the plasma samples as an internal standard. Samples were injected onto a μ Bondapak C₁₈ Radial-Pak column (Waters), followed by equilibration in a mobile phase consisting of 4 percent acetonitrile in 0.1 M monobasic potassium phosphate buffer (pH 2.1).

Determination of DNA Methylation

DNA methylation status was assessed by measuring the *in vitro* incorporation of methyl groups into global and CpG island DNA as described by Pogribny *et al.* (215), with minor modifications. DNA was purified from liver samples (186) using a commercial kit (Promega). The DNA (1.25 μ g) was then digested overnight using HpaII endonuclease for global DNA and BSHII endonuclease for CpG island digestion (New England Biolabs Inc.). The assay mixture consisted of 1.0 μ g digested DNA, 1X PCR Buffer II, 1mM magnesium chloride, 0.5 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), and 9.3 μ M [³H]-dCTP (NEN Life Science Products, Boston, MA) in a total volume of 40 μ L.

Following a one hour incubation period at 55°C, samples were applied to DE81 ion exchange filter paper, washed with 0.5 M sodium phosphate buffer, dried, and [³H]-dCTP incorporation was quantified via liquid scintillation counting. The extent of [³H]-dCTP incorporation is directly proportional the number of original unmethylated sites (215).

MS Activity Analysis

The activity of MS was determined as previously described by Keating *et al.* (134). The reaction mixture contained 500 mM sodium phosphate buffer (pH 7.5), 1.3 μM cyanocobalamin, 1 M dithiothreitol, 10 mM SAM, 82.4 mM β-mercaptoethanol, 100 mM homocysteine, and 15 mM [*methyl*-¹⁴C]-THF (0.17 μCi/μmol). This mixture was added to liver supernatants and incubated for one hour at 37°C. Ice-cold water was added to terminate the reaction and the assay mixture was immediately applied to an AG 1-X8 (chloride form) resin column. Effluent fractions (3 mL deionized water) were collected for subsequent liquid scintillation counting. Homocysteine was prepared fresh daily from a thiolactone derivative.

PEMT Activity Analysis

PEMT activity was determined by measuring the incorporation of radiolabeled methyl groups from S-adenosyl-L-[methyl-³H]methionine into phospholipids according to the method of Duce *et al.* (74) with modifications. Briefly, the reaction mixture contained 10 mM HEPES (pH 7.3), 4 mM dithiothreitol, 5 mM MgCl₂, 0.1 mM SAM, 2 μCi S-adenosyl-L-[methyl-³H]methionine, and 750 μg protein in a final volume of 550 μL. Exogenous PE was not added to the reaction mixture since because it has not been shown to significantly increase the reaction rate (34, 111). The reaction was initiated by adding 750 μg microsomal protein and incubated in a 37°C water bath for 10 minutes. 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 percent (v/v) methanol, transferred to glass scintillation vials, and allowed to dry at room temperature. The lipid fraction was reconstituted in scintillation fluid and the radioactivity was determined by liquid scintillation counting.

Statistical Analysis

The mean values of each treatment group were analyzed by a two-way ANOVA test. When the variance between means was found to be significant, mean values were compared

using Fisher's least significant difference procedure. GNMT activity and total homocysteine were both compared with the blood glucose levels using the Pearson correlation analysis (254). ANOVA, Fisher's least significant difference procedure, and Pearson's correlation analysis were performed using SigmaStat software and a significance level of 5 percent.

Results

Cumulative weight gain is not affected by insulin treatment in diabetic rats; however blood glucose concentrations are restored. Diabetic rats gained approximately 50 percent less weight than control rats (Table 5.1). Insulin-treated diabetic rats gained approximately 40 percent more weight than diabetic rats; however, this gain did not reach statistical significance ($p = 0.07$). STZ was effective at inducing a diabetic state in non-fasted rats evident by a 2.9-fold increase in blood glucose concentrations. Further, insulin treatments effectively reduced blood glucose concentrations in non-fasted diabetic rats to 50 percent of concentrations exhibited in diabetic rats that received only saline injections.

GNMT induction in diabetic animals is prevented by insulin treatment, and positively correlated with blood glucose concentrations. Hepatic GNMT activity was elevated 1.6-fold in STZ-induced diabetic rats, compared to control rats (**Figure 3.2A**). However, insulin treatments in diabetic rats restored activity levels to that of control animals, reaching only 70 percent of activity levels exhibited by diabetic rats. Similarly, activity levels were reflected in abundance of the protein (**Figure 3.2B**). GNMT abundance was 1.9-fold greater in STZ-induced diabetic animals, as compared the control group. Again, insulin prevented this induction in diabetic rats, reaching only 75 percent of abundance levels evident in diabetic animals that received only saline. Moreover, when plotting GNMT activity against blood glucose concentrations for each animal, a positive correlation was established ($r = 0.80$, $p = 0.0001$, **Figure 3.3**).

Insulin treatment reverses hypohomocysteinemia in diabetic rats, and negatively correlates with blood glucose concentrations. Total plasma homocysteine concentrations were reduced 2.3-fold by STZ-induced diabetes, compared to the control group (**Figure 3.4**). Insulin treatments seemed to prevent hypohomocysteinemia in diabetic rats, elevating plasma homocysteine levels 2.8-fold over diabetic rats that did not receive insulin. When total

plasma homocysteine concentrations were plotted against blood glucose concentrations for each rat, a negative correlation was evident ($r = -0.30$, $p = 0.10$, **Figure 3.5**)

Both global and CpG island hepatic DNA hypomethylation were restored by insulin treatments in diabetic animals. Hepatic global DNA methylation was reduced 4.7-fold in diabetic animals, compared to the control group (**Figure 3.6**). Similarly, a diabetic state also induced hepatic CpG hypomethylation, where DNA methylation was reduced 1.4-fold as compared to control animals. Insulin treatments returned methylation status to levels comparable to that of control animals. Hypomethylation in insulin-treated diabetic rats reached only 24 and 68 percent of the CpG and global hypomethylation status exhibited by diabetic rats, respectively.

Hepatic MS and PEMT activity are altered in diabetic animals but returned in insulin-treated diabetic rats. As seen previously, hepatic activity of folate-dependent remethylation enzyme MS is dramatically reduced (2.5-fold) by an STZ-induced diabetic state, as compared to control animals (**Table 3.2**). However, with the addition of insulin injections, MS activity increased 2.4-fold to within the activity level of the control group. Conversely, a diabetic state increased hepatic PEMT activity 1.5-fold. Again, this increase in PEMT activity was prevented with insulin injections in diabetic animals, where activity levels reached only 70 percent of activity levels exhibited by diabetic animals. Interestingly, PEMT activity was also positively correlated with blood glucose concentrations ($r = 0.40$, $p = 0.80$, **Figure 3.7**)

Discussion

The disruption of homocysteine and methyl group metabolism has been repeatedly linked to numerous pathologies. It is subsequently vital to maintain normal methyl group metabolism and homocysteine homeostasis, as well as identify those factors that may disrupt these metabolic pathways for optimal health. We and others have clearly established that a diabetic state disrupts homocysteine and methyl group metabolism. Specifically a type 1 diabetic state seemingly results in a functional methyl group deficiency owing to the induction of PEMT, GNMT, and reduced MS activity (101, 197, 198). Type 1 diabetics typically maintain blood glucose concentrations with insulin injections. Insulin treatment has reportedly reversed the induction of homocysteine transsulfuration and folate-independent remethylation in streptozotocin-induced diabetic rats and cells treated with glucocorticoids

(123, 219, 220). We have also shown *in vitro*, that pretreatment with insulin prevents glucocorticoid-induced GNMT induction (198). GNMT has been established as regulator of methyl group supply since it is (i) abundant, (ii) less sensitive to SAH inhibition compared to other methyltransferases, (iii) its product sarcosine has no known physiological function, and (iv) its activity is inhibited by 5-CH₃THF (135, 289, 291, 310). As a result the present study was aimed at determining if insulin could also maintain normal methyl group metabolism, namely GNMT, which is otherwise perturbed by STZ-induced diabetes.

Untreated STZ-treated rats were clearly diabetic in our study as indicated by a 3-fold increase in blood glucose concentration as compared to control levels. Insulin treatments effectively returned blood glucose levels to within normal values. Plasma homocysteine levels were significantly reduced in diabetic animals and restored by insulin treatment, as previously reported (123). Hypohomocysteinemia, in diabetic humans and animals with normal renal function, typically assessed by creatinine levels, is commonly reported and thought to be the result of increased transsulfuration of the amino acid as well as increased folate-independent remethylation (123, 198, 220, 224, 299). However, as kidney function deteriorates, homocysteine disposal becomes impaired, consequently homocysteine levels rise (271, 305).

We have expanded on previous knowledge, showing that STZ-induced GNMT and PEMT induction and DNA hypomethylation were prevented by insulin treatment, conceivably restoring methyl group supply. PEMT is considered a major consumer of methyl groups from SAM, thus a major contributor to homocysteine production (256). However elevated PEMT in diabetic rats was not correlated with elevated plasma homocysteine levels, likely due to increased transsulfuration and folate-independent remethylation in STZ-induced diabetic rats (123, 197, 220). Moreover, GNMT and PEMT activity levels positively correlated and homocysteine concentrations negatively correlated with blood glucose concentrations. This supports a role for insulin or glucose in the regulation of these enzymes possibly at the level of transcription. This speculation can be further supported by a lack of response to treatment that was exhibited by a few animals, as evident by their blood glucose concentrations, yet removal of these data points did not improve the correlation. It will be critical in future studies to assess mRNA abundance and

elucidate a mechanism for the protection provided by insulin. However we have previously reported that GNMT mRNA abundance is upregulated in a diabetic state and further actinomycin D, a potent inhibitor of transcription, prevents induction of GNMT by glucocorticoids *in vitro* (197)(Rowling and Schalinske, unpublished data). Additionally, insulin may regulate gene transcription of other additional enzymes involved in homocysteine and methyl group metabolism. More specifically insulin decreased BHMT gene transcription rate and in turn mRNA abundance in rat hepatoma cells (220). Insulin also prevented CBS mRNA levels elevated by glucocorticoids and STZ-induced diabetes (123, 219).

In agreement with previous studies (198, 220), hepatic SAM and SAH were significantly elevated in STZ-induced diabetic rats (304.1 ± 117.3 versus 24.2 ± 3.3 and 188.3 ± 33.8 versus 77.6 ± 4.6 , respectively) and returned with insulin treatments (18.6 ± 5.1 and 83.0 ± 8.5 , respectively, data not shown). Hepatic SAM levels are also elevated in a type 2 diabetic rat model without renal complications (299) and in the glucagon-treated rat (124). In contrast to Ratnam *et al.* (220), the SAM:SAH ratio was also elevated in diabetic rats and returned to normal levels by insulin administration. In support of this previous reports indicate the SAM:SAH ratio is also elevated in STZ-induced diabetic rats, however the trend did not reach significance ($p=0.1$)(198). However, the degree of impaired renal function in type 2 diabetics has been positively associated with plasma SAH and homocysteine concentrations and negatively correlated with the SAM:SAH ratio (108). Thus our treatment period may have been insufficient for elevating SAH and decreasing the SAM:SAH ratio.

It is plausible that SAM may also serve as a regulator in response to hormones. Interestingly elevated MAT activity and mRNA abundance have been reported in glucocorticoid-treated rats (94) and a rat model of type 2 diabetes (299). SAM is considered a regulator of methyl group metabolism, as it allosterically activates CBS, committing homocysteine to transsulfuration to manage accumulation (82). Thus elevated SAM in diabetic animals may be promoting increased transsulfuration and the characteristic reduction in homocysteine levels in diabetic animals and humans with normal renal function. SAM also negatively regulates MTHFR, decreasing production of 5-CH₃THF and removing inhibition of GNMT. This may explain both the reduction in folate-independent

remethylation via MS and increased GNMT activity in diabetic animals. Further PEMT activity has been reportedly increased following methionine infusion and subsequently elevated SAM and the SAM:SAH ratio (286). The SAM:SAH ratio was not reduced, which would have been a likely explanation for DNA hypomethylation since SAH is a potent inhibitor of methyltransferases. However, since PEMT and GNMT activity are elevated and methyl groups from the one-carbon pool are reduced, a functional methyl group deficiency may exist in diabetic animals; ultimately decreasing methyl groups for DNA methylation. In support of this explanation, hypomethylation was also reported in rats treated with retinoic acid, which also induced GNMT (229). Further, DNA hypomethylation has also been reported in folate deficiency (19, 296).

In summary, we have shown that disruption of methyl group metabolism disrupted by a type 1 diabetic state was prevented by insulin. This provides further insight for insulin or glucose as a regulator of homocysteine and methyl group metabolism, and stresses the importance of blood glucose control in diabetics. Importantly similar disturbances have been reported in type 2 diabetic rat models (220) and humans with insulin resistance (226). Thus future research needs to be aimed at more closely identifying the protection provided by insulin in maintaining these important metabolic pathways.

Footnotes

¹ Hartz CS³, Nieman KM³, Jacobs RL⁴, Vance DE⁴, Schalinske KL³. Hepatic phosphatidylethanolamine *N*-methyltransferase and homocysteine metabolism in diabetic rats. *J Nutr* 136(12): 3005-3009, 2006.

² Nieman KM, Schalinske KL. Modulation of methyl group and homocysteine metabolism by insulin treatment in diabetic rats. *FASEB J* 20(4): A607, 2006 (abstr.).

³ Graduate students and associate professor, Interdepartmental Graduate Program in Nutritional Sciences and the Department of Food Science and Human Nutrition, Iowa State University.

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Tables

Table 3.1

Cumulative weight gain and blood glucose concentrations in streptozotocin (STZ)-induced diabetic rats with or without insulin (INS) treatment¹

	Cumulative Weight Gain (g)	Blood glucose (mM)
Control	60 ± 2^a	9.0 ± 1.0^a
STZ	29 ± 4^b	26.1 ± 1.3^b
STZ+INS	42 ± 9^b	13.2 ± 2.8^a

¹Values are means \pm S.E. (n = 4-6). Data in each column with distinct letters are significantly different ($p \leq 0.05$).

Table 3.2

Hepatic enzyme activity of methionine synthase (MS) and phosphatidylethanolamine N-methyltransferase (PEMT) in insulin (INS)-treated diabetic rats induced by streptozotocin (STZ)¹

	MS (pmol/min•mg protein)	PEMT (pmol/min•mg protein)
Control	56 ± 5 ^a	36 ± 5 ^a
STZ	22 ± 4 ^b	55 ± 5 ^b
STZ+INS	52 ± 9 ^b	38 ± 3 ^a

¹Values are means ± S.E. (n = 4-6). Data in each column with different letters are significantly different ($p \leq 0.05$).

Figure Legends

Figure 3.1

Folate, homocysteine, and methyl group metabolism. Methionine provided via the diet or endogenously following remethylation of homocysteine is activated, with the addition of adenosine, to *S*-adenosylmethionine (SAM). This reaction, requiring ATP, is catalyzed by the enzyme methionine adenosyltransferase (MAT). SAM is the primary methyl group donor in numerous transmethylation reactions. The donation of a methyl group by SAM in various methylation reactions ($X \rightarrow X\text{-CH}_3$) results in *S*-adenosylhomocysteine (SAH). The activity of phosphatidylethanolamine *N*-methyltransferase (PEMT), a major consumer of methyl groups from SAM, results in the production of phosphatidylcholine (PC) from phosphatidylethanolamine (PE). Since SAH is a potent inhibitor of most transmethylation reactions it is critical to regulate the SAM:SAH ratio, which is accomplished by glycine *N*-methyltransferase (GNMT). This abundant cytosolic protein accomplishes this control by utilizing methyl group from SAM in the conversion of glycine to sarcosine. SAH is hydrolyzed to the non-protein forming amino acid homocysteine by SAH hydrolase (SAHH). Homocysteine exists at a branch point in these interrelated metabolic pathways. The amino acid can be irreversibly catabolized to cysteine through transsulfuration or remethylated, restoring methionine pools. Homocysteine transsulfuration to cysteine relies on two B₆-dependent enzymes cystathionine β-synthase (CBS) and γ-cystathionase. Remethylation of homocysteine occurs by both folate-independent and folate-dependent pathways. Betaine, formed following the oxidation of choline, provides a methyl group in folate-independent remethylation. Dimethylglycine (DMG) and methionine result from the activity of betaine-homocysteine *S*-methyltransferase (BHMT). Folate-dependent remethylation functions through the donation of a methyl group by 5-methyltetrahydrofolate (5-CH₃THF). The B₁₂-dependent enzyme methionine synthase (MS) catalyzes this reaction forming tetrahydrofolate (THF) and methionine. THF utilizes a carbon from serine generating 5,10-methylenetetrahydrofolate (5,10-CH₂THF), which is irreversibly reduced to 5-CH₃THF by the B₂-dependent enzyme 5,10-CH₂THF reductase (MTHFR).

Figure 3.2

Hepatic glycine *N*-methyltransferase (GNMT) activity and protein levels are restored by insulin in streptozotocin (STZ)-induced diabetic and insulin-treated diabetic rats (STZ+INS). Rats were acclimated to their diet and surroundings for a 6 day period. At the end of the acclimation period rats received a single intraperitoneal injection of STZ (60 mg/kg body weight) or the vehicle 10 mM citrate buffer (pH 4.5). The following day rats were given INS injections (1.0 unit/200 μ L saline) or the vehicle twice daily for 5 days. Liver was collected from these animals and GNMT activity and abundance were determined as described under “Materials and Methods.” **A:** Hepatic GNMT activity in diabetic and insulin-treated diabetic rats. Data are expressed as means \pm S.E. ($n = 4-6$), where an asterisk (*) indicates a statistically significant difference ($p \leq 0.05$). **B:** GNMT abundance was assessed by Western blot analysis using a monoclonal antibody. Data are expressed as means \pm S.E. ($n = 4-6$) and an asterisk (*) denotes a significant difference ($p \leq 0.05$). Data are displayed as a percent of the control group mean in addition to a representative blot.

Figure 3.3

Hepatic glycine *N*-methyltransferase (GNMT) activity positively correlates with blood glucose concentrations in diabetic and insulin-treated diabetic rats. Hepatic GNMT activity data was plotted against blood glucose concentration for the same animals as described for Figure 3.2 and within the “Materials and Methods” section. Each treatment group is represented by a distinct symbol: control, ●; streptozotocin (STZ)-induced diabetic, ■; insulin-treated diabetic (STZ+INS), ◆. A trend line was drawn which best fit the data and the correlation statistics are noted on the graph.

Figure 3.4

Insulin restores plasma homocysteine concentrations in diabetic rats. Plasma was collected from the same animals described for Figure 3.2: control, streptozotocin (STZ)-induced diabetic, and insulin treated diabetic (STZ+INS) rats. Total homocysteine was quantified using HPLC with fluorometric detection as describe in the “Materials and Methods” section. Data are expressed as means \pm S.E. ($n = 4-6$) with and asterisk (*) indicating a significant difference ($p \leq 0.05$).

Figure 3.5

Total plasma homocysteine negatively correlates with blood glucose concentrations in diabetic and insulin-treated diabetic rats. Plasma homocysteine data was plotted against blood glucose concentration for the same animals as described for Figure 3.2 and within the “Materials and Methods” section. Each treatment group is represented by a distinct symbol: control, ●; streptozotocin (STZ)-induced diabetic, ■; insulin-treated diabetic (STZ+INS), ◆. A trend line was drawn which best fit the data and the correlation statistics are noted on the graph.

Figure 3.6

Hypomethylation of hepatic global and CpG island DNA is reversed by insulin (INS) treatment in streptozotocin (STZ)-induced diabetic rats. Liver was collected from the same animals as described for Figure 3.2 for DNA isolation. Methylation status was assessed using [³H]-dCTP, where incorporation of radiolabeled dCTP is directly proportional to unmethylated sites as described in the “Materials and Methods” section. Data are expressed as means ± S.E. and presented as a percent of the respective control group mean. Bars denoted with an asterisk (*) indicate a significant difference ($p \leq 0.05$).

Figure 3.7

Hepatic phosphatidylethanolamine *N*-methyltransferase (PEMT) activity positively correlates with blood glucose concentrations in diabetic and insulin-treated diabetic rats. Hepatic PEMT activity data was plotted against blood glucose concentration for the same animals as described for Figure 3.2 and within the “Materials and Methods” section. Each treatment group is represented by a distinct symbol: control, ●; streptozotocin (STZ)-induced diabetic, ■; insulin-treated diabetic (STZ+INS), ◆. A trend line was drawn which best fit the data and the correlation statistics are noted on the graph.

Figures

Figure 3.1

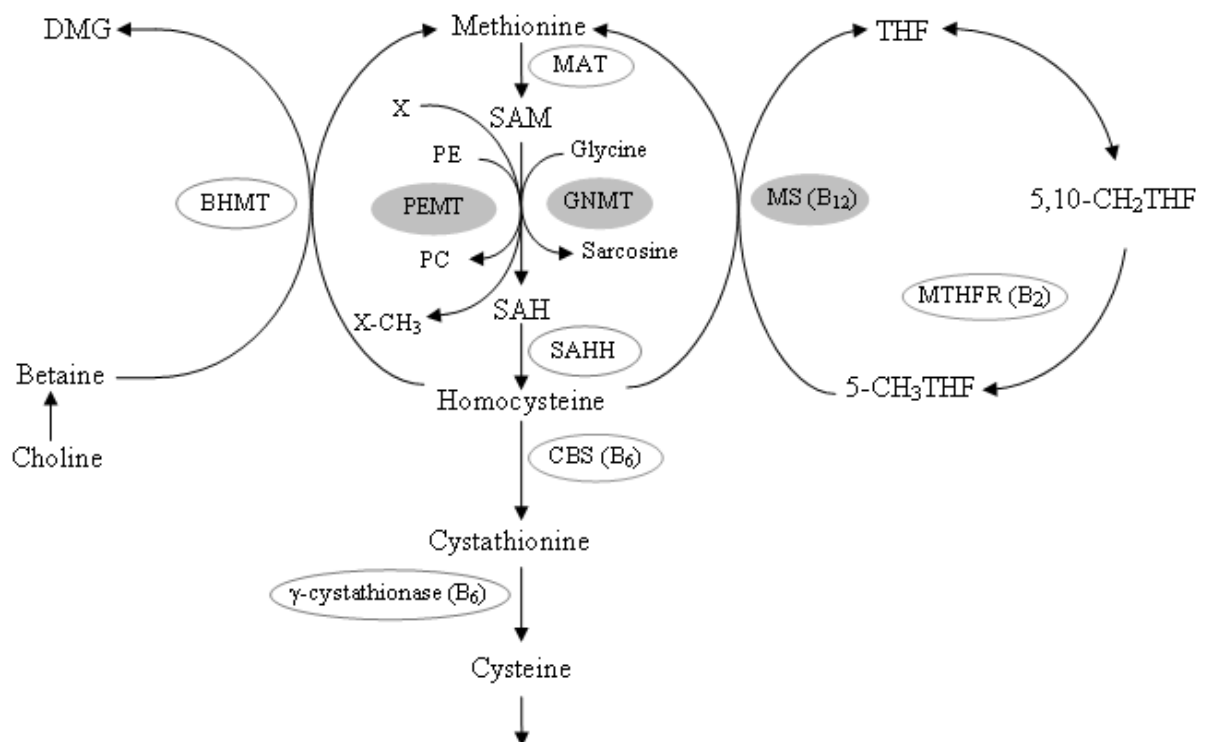


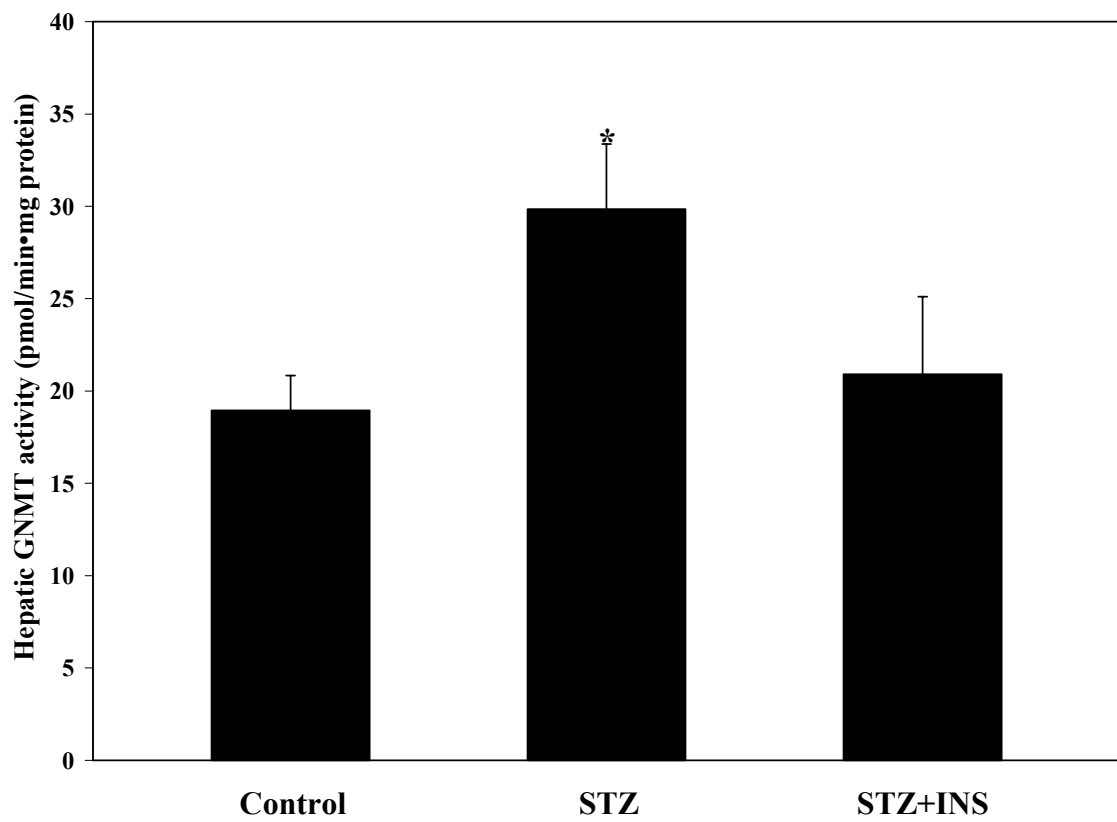
Figure 3.2A

Figure 3.2B

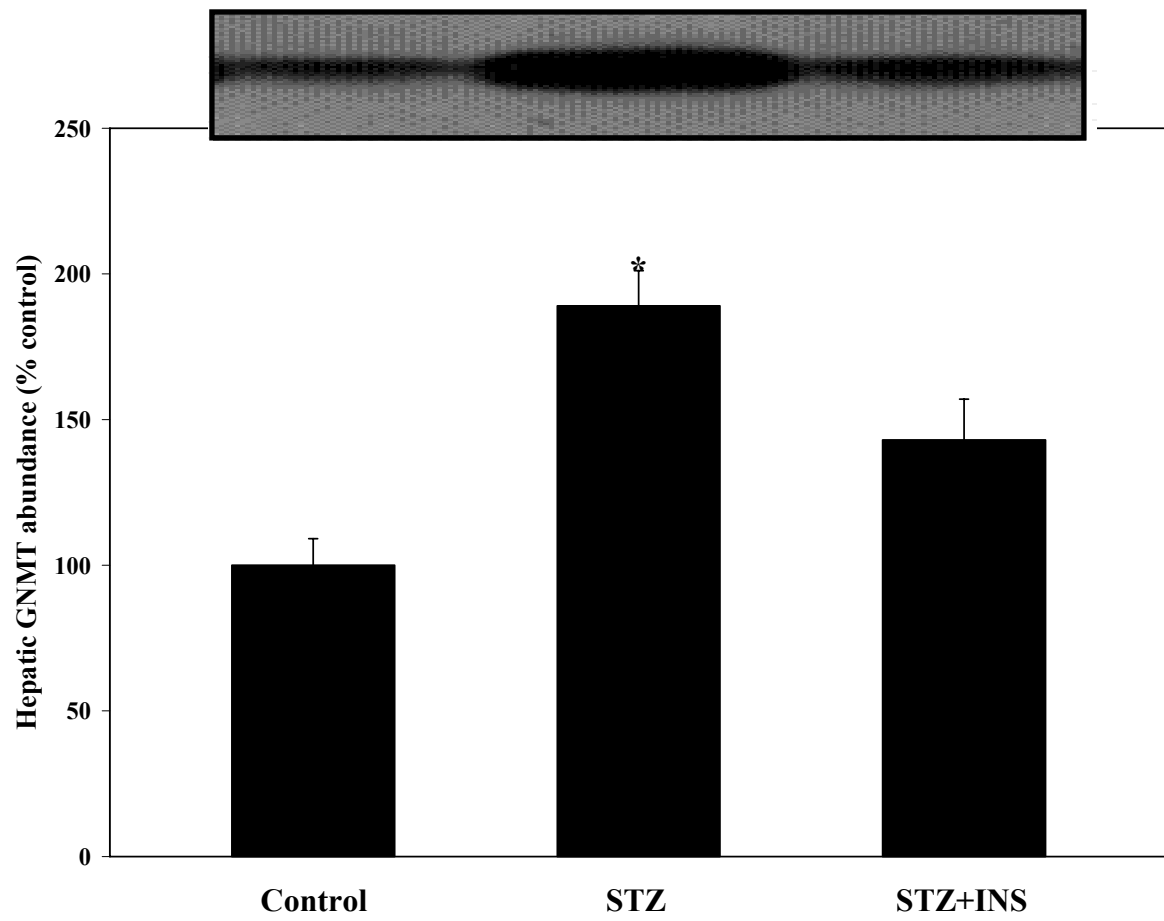


Figure 3.3

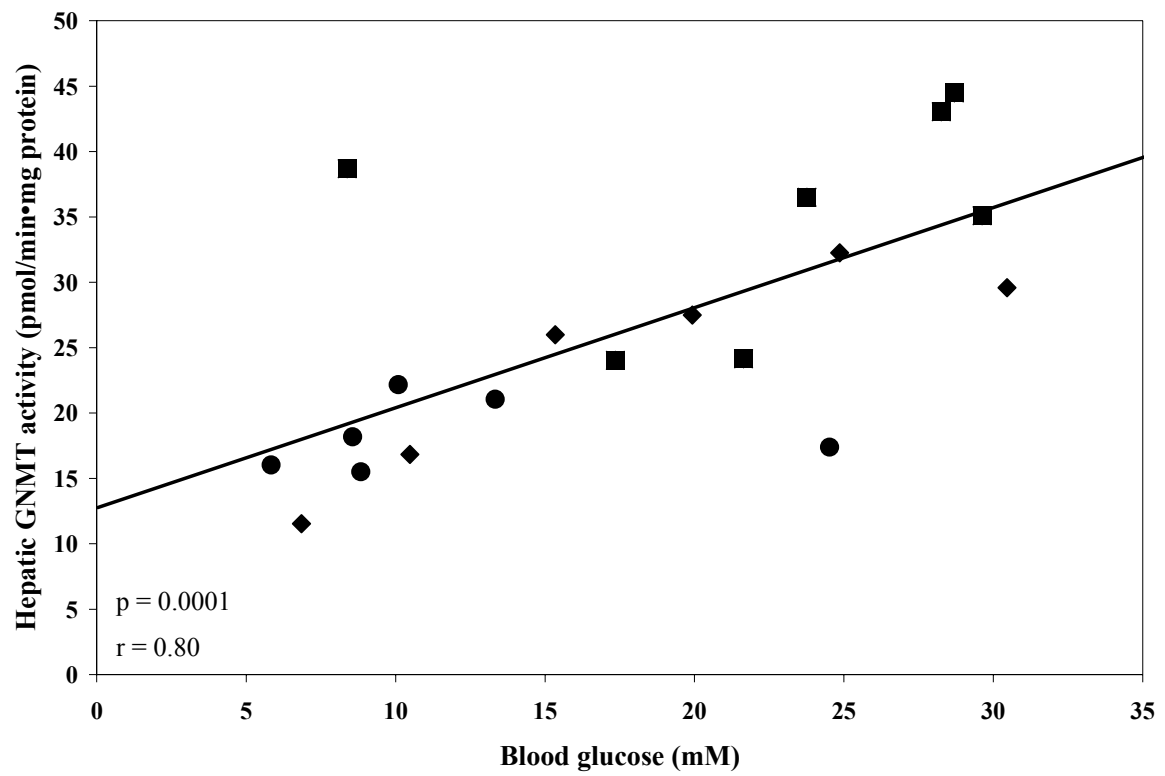


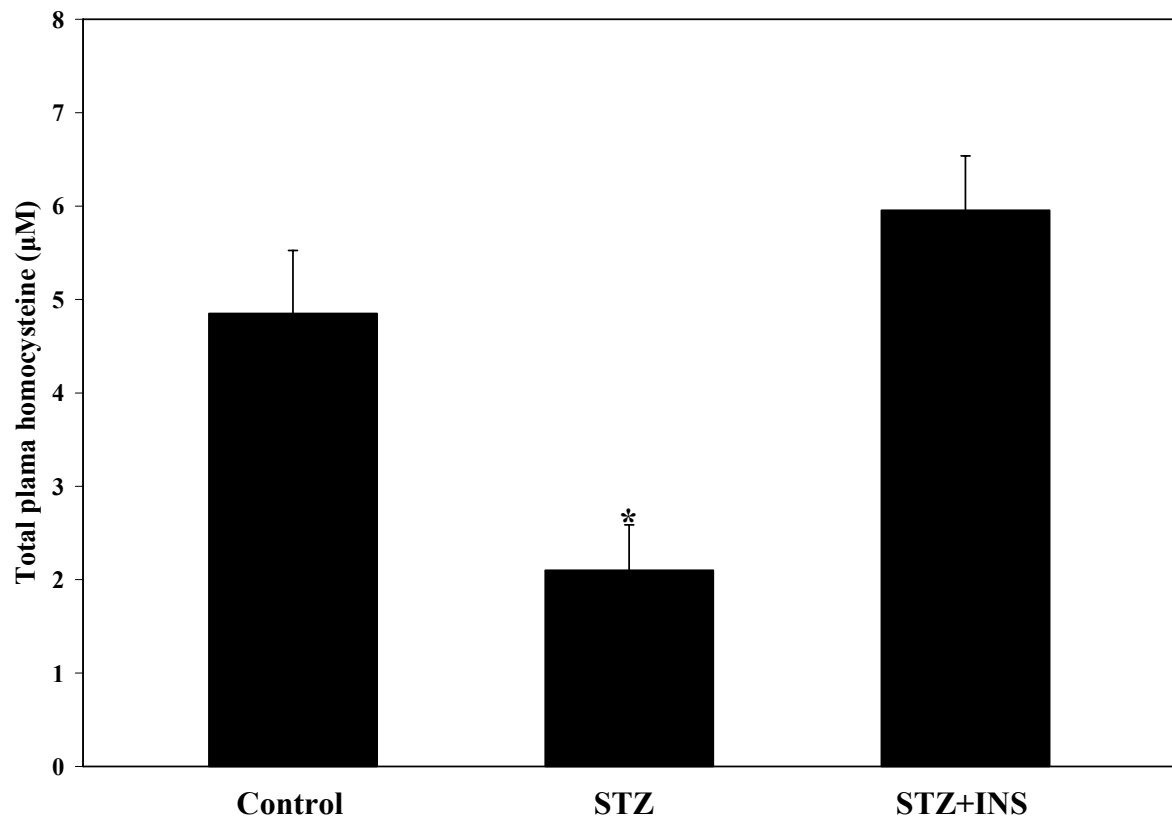
Figure 3.4

Figure 3.5

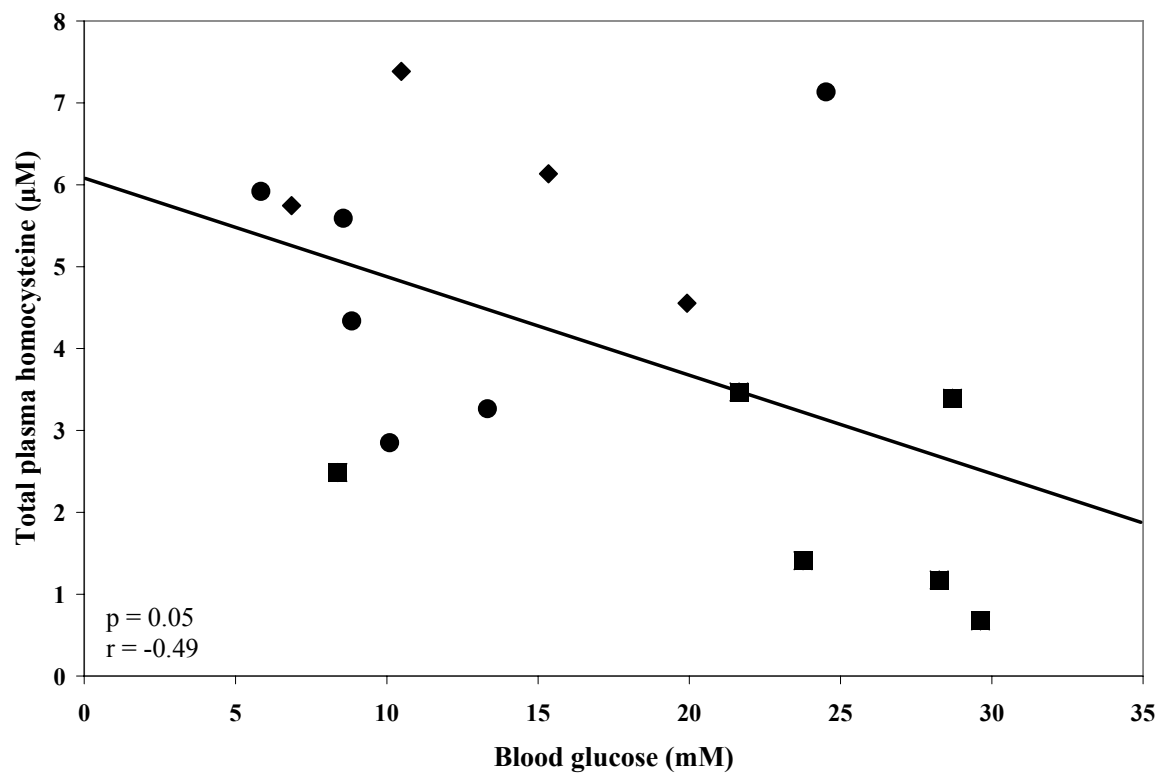


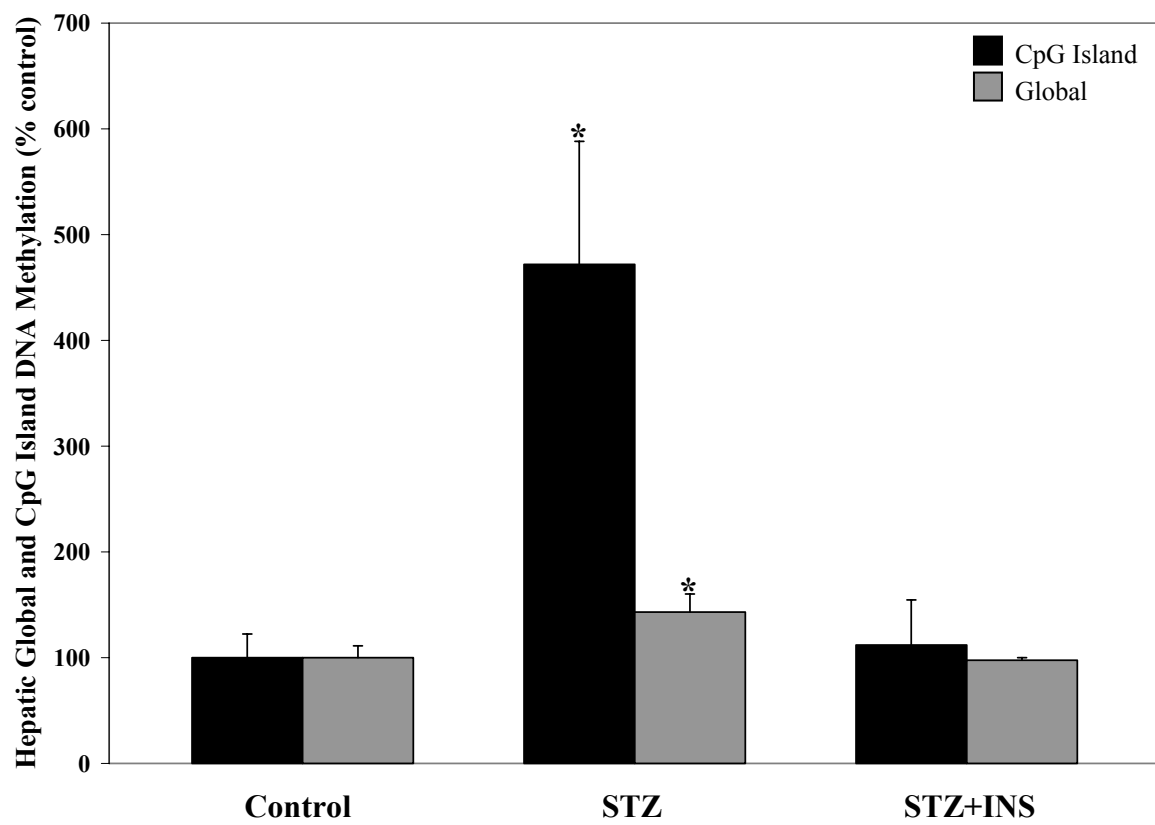
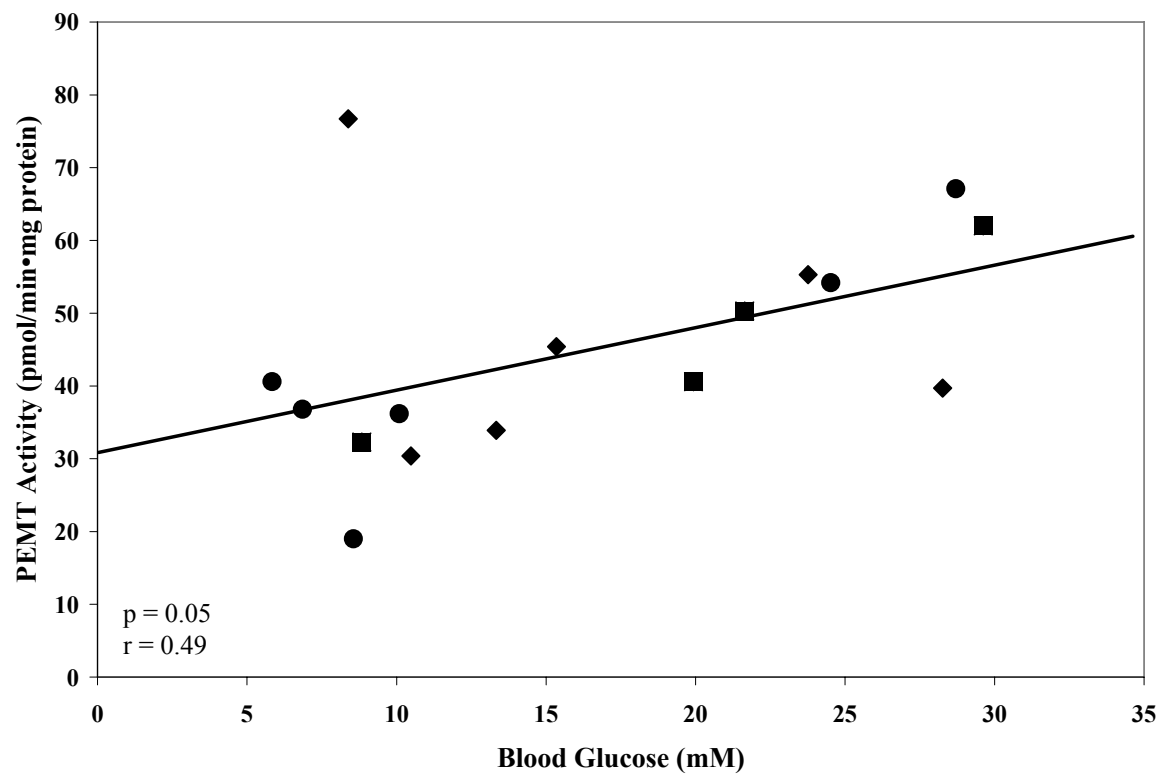
Figure 3.6

Figure 3.7



CHAPTER 4: FOLATE STATUS MODULATES THE INDUCTION OF HEPATIC GLYCINE *N*-METHYLTRANSFERASE AND HOMOCYSTEINE METABOLISM IN DIABETIC RATS¹

Modified from a paper published in the *American Journal of Physiology-Endocrinology and Metabolism*¹ and an abstract published in the *Federation of American Societies for Experimental Biology Journal*²

Abstract

A diabetic state induces the activity and abundance of glycine *N*-methyltransferase (GNMT), a key protein in the regulation of folate, methyl group, and homocysteine metabolism. Because the folate-dependent one-carbon pool is a source of methyl groups and 5-methyltetrahydrofolate allosterically inhibits GNMT, the aim of this study was to determine if folate status has an impact on the interaction between diabetes and methyl group metabolism. Rats were fed a diet containing deficient (0 ppm), adequate (2 ppm), or supplemental folate (8 ppm) for 30 days, after which diabetes was initiated in half of the rats by streptozotocin treatment. The activities of GNMT, phosphatidylethanolamine *N*-methyltransferase (PEMT), and betaine homocysteine *S*-methyltransferase (BHMT) were increased approximately 2-fold in diabetic rat liver; folate deficiency resulted in the greatest elevation in GNMT activity. The abundance of GNMT protein and mRNA, as well as BHMT mRNA, were also elevated in diabetic rats. Hyperhomocysteinemia in folate-deficient rats was attenuated by streptozotocin, likely due in part to increased BHMT expression. These results indicate that a diabetic state profoundly modulates methyl group, choline, and homocysteine metabolism, and folate status may play a role in the extent of these alterations. Further, the up-regulation of BHMT and PEMT may indicate an increased choline requirement in the diabetic rat.

Introduction

The folate-dependent one-carbon pool and methyl group metabolism are interrelated pathways critically important in optimal health, as perturbation of these metabolic processes is associated with a number of pathologies including cardiovascular disease, cancer

development, and birth defects (**Figure 4.1**) (132, 227, 240). The primary methyl group donor, *S*-adenosylmethionine (SAM), requires a constant supply of methyl groups from the diet and/or the one-carbon pool for numerous transmethylation reactions, such as the synthesis of phosphatidylcholine (PC) by the action of the liver-specific enzyme phosphatidylethanolamine *N*-methyltransferase (PEMT) (176). Therefore, it is essential to regulate the supply and utilization of methyl groups to optimize SAM-dependent transmethylation reactions, a function that is accomplished by the enzymatic activity of a key regulatory protein, glycine *N*-methyltransferase (GNMT). GNMT is an abundant protein in the liver, comprising approximately 1-3 percent of all hepatic cytosolic protein, and has also been identified in renal and pancreatic tissue (205, 310). GNMT optimizes the SAM/ *S*-adenosylhomocysteine (SAH) ratio by catalyzing the conversion of SAM and glycine to SAH and sarcosine, respectively (41, 42, 103). Because SAH is a potent inhibitor of methyltransferase activity (135), optimizing the SAM/SAH ratio serves to regulate the transmethylation capacity of the cell (289).

Following SAM-dependent transmethylation and the hydrolysis of SAH by SAH hydrolase (SAHH), the resulting homocysteine can be remethylated back to methionine or further catabolized through the transsulfuration pathway to cysteine by the initial action of cystathionine β -synthase (CBS). Remethylation occurs through both folate-dependent and folate-independent pathways, both contributing equally to methionine regeneration (85). The folate-independent remethylation route utilizes betaine, derived from the oxidation of choline, and the enzymatic action of betaine homocysteine *S*-methyltransferase (BHMT) that results in the formation of dimethylglycine and methionine (81). Alternatively, homocysteine can be remethylated through the donation of a methyl group from 5-methyltetrahydrofolate (5-CH₃-THF) via the B₁₂-dependent enzyme methionine synthase (MS), resulting in tetrahydrofolate (THF) and methionine. An inability of the cell to remethylate or catabolize homocysteine can result in hyperhomocysteinemia, an independent risk factor for cardiovascular disease (55, 132).

Because of its central role in methyl group and homocysteine metabolism, the regulation of GNMT is an important control point. In a state of excess methyl groups, the resulting increase in SAM acts as an allosteric inhibitor of the enzyme 5,10-methylenetetrahydrofolate

reductase (MTHFR) (127, 149), decreasing its activity and the subsequent production of 5-CH₃-THF. This alleviates the negative allosteric regulation 5-CH₃-THF imposes on GNMT, thereby increasing its activity and disposing of the excess methyl groups. Conversely, a lack of methyl groups results in an increase in MTHFR activity and 5-CH₃-THF concentrations, thereby inhibiting GNMT and conserving methyl groups for transmethylation reactions (291).

A *type 1* diabetic state has been identified as a pathological factor in the modulation of methyl group and homocysteine metabolism. A reduction in circulating concentrations of homocysteine as a result of increased catabolism through the transsulfuration pathway, namely by the transcriptional regulation of CBS, has been demonstrated in a streptozotocin (STZ)-induced diabetic rat model (123, 219). Recently, we have shown that a diabetic state leads to the disruption of hepatic methyl group metabolism, characterized by elevations in GNMT activity and abundance, as well as an increase in the folate-independent remethylation of homocysteine by BHMT (198). Similarly, administration of specific counter-regulatory hormones to insulin (e.g. dexamethasone and glucagon) have also been shown to alter methyl group and homocysteine metabolism both *in vivo* and *in vitro* (124, 230). Because the folate-dependent one-carbon pool supplies methyl groups for the remethylation of homocysteine and SAM-dependent transmethylation reactions, and serves as a regulatory mechanism for the control of GNMT activity via allosteric inhibition by 5-CH₃-THF, the aim of this study was to examine how dietary folate status may impact the previously reported findings. We have also extended our earlier studies by examining the activity of PEMT, because it is a key enzyme in the SAM-dependent synthesis of PC and regulation of homocysteine homeostasis (200, 249).

Materials and Methods

Chemicals and Reagents

Reagents used in the research methods were obtained from the following sources: S-adenosyl-L-[*methyl*-³H]methionine, PerkinElmer Life Sciences; chemiluminescent Western blotting detection reagents, Amersham Biosciences; streptozotocin and S-adenosyl-L-methionine, Sigma-Aldrich; goat anti-mouse IgG horseradish peroxidase, Southern Biotechnology. Dr. Yi-Ming Chen of National Yang-Ming University, Taipei Taiwan,

generously provided the GNMT antibody (155). All other chemicals were of analytical grade.

Animals

All experiments involving animals were approved by and conducted in accordance with the Iowa State University Laboratory Animal Resources guidelines. Male Sprague-Dawley rats (50-74 grams) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and housed in separate cages with a 12-h light: dark cycle. Animals were allowed access to food and water *ad libitum*. Rats were randomly assigned to treatment groups and fed an amino-acid defined diet (# TD.03333, Harlan Teklad, Madison, WI, **Table 4.1**) 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, non-fasted rats were anesthetized 3-4 hours into the light cycle with a single intraperitoneal injection of ketamine:xylazine (90:10 mg/kg body weight) and heparinized whole blood was collected by cardiac puncture. An aliquot was removed for the determination of blood glucose concentrations using a commercial kit (Sigma-Aldrich); the remaining whole blood sample was centrifuged at $4,000 \times g$ for 6 min and the plasma was removed for storage at -20°C for subsequent analysis of homocysteine concentrations. Portions of the liver were rapidly removed and homogenized in four volumes of ice-cold buffer containing 10 mM sodium phosphate (pH 7.0), 0.25 M sucrose, 1 mM EDTA, 1 mM sodium azide, and 0.1 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at $20,000 \times g$ for 30 minutes and the resulting supernatant was stored at -70°C after the addition of β -mercaptoethanol to a final concentration of 1 mM. These samples were used for the determination of enzyme activities (GNMT, BHMT, MS) and abundance (GNMT). A second liver sample was homogenized in 4 volumes of ice-cold sodium acetate buffer (100 mM, pH 4.9) containing 0.5 percent ascorbate (w/v) and 20 mM 2-mercaptoethanol under a steady stream of nitrogen, tightly capped, and stored at -70°C for determination of hepatic folate concentrations. Additional whole liver samples were placed in liquid nitrogen or RNeasyTM (Qiagen) and stored at -70°C for subsequent isolation of microsomes (PEMT) or mRNA (GNMT, BHMT), respectively. Total soluble protein concentration of the

supernatants was determined using a commercial kit (Coomassie Plus, Pierce) based on the Bradford method (32) and bovine serum albumin as a standard. All enzyme assays were linear with respect to protein concentration and incubation time.

Determination of GNMT Activity and Abundance

The enzymatic activity of GNMT was measured based on the method of Cook and Wagner (60) with slight modifications (230). 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*-³H]methionine. Abundance of the GNMT protein was determined by immunoblotting methods described previously (230) using a 10-20 percent (w/v) gradient SDS-polyacrylamide gel to separate the 32-kDa monomer subunit of the protein. Proteins were transferred to nitrocellulose paper and incubated with a 1: 4,000 dilution of the monoclonal GNMT antibody (155) 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 one hour at room temperature. Proteins were detected using chemiluminescence and band density was determined using SigmaGel software (SPSS, Chicago, IL).

Determination of PEMT Activity

Approximately one gram 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 $16,000 \times g$ for 20 minutes at 4°C and 1-2 mL of the resulting supernatant was centrifuged at $105,000 \times g$ for 60 minutes at 4°C. The microsomal pellet was resuspended in 400 µL of 0.25 M sucrose for determination of PEMT activity by measuring the incorporation of radiolabeled methyl groups from *S*-adenosyl-L-[*methyl*-³H]methionine into phospholipids according to the method of Duce *et al.* (74) with modifications. This specific assay using endogenous phosphatidylethanolamine (PE) has been shown to accurately determine PEMT activity equivalent to methods that add exogenous PE to the incubation mixture (34, 111). Briefly, the reaction mixture contained 10 mM HEPES (pH 7.3), 4 mM dithiothreitol, 5 mM MgCl₂, 0.1 mM SAM, 2 µCi *S*-adenosyl-L-[*methyl*-³H]methionine, and 750 µg protein in a final volume of 550 µL. The reaction was initiated by adding 75 µL microsomal protein and incubated in a 37°C water bath for 10 minutes. 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 M KCl in 50 percent methanol, transferred to glass scintillation vials, and allowed to dry at room temperature. The lipid fraction was reconstituted in 5 mL Scintiverse[®] (Fisher Scientific, Pittsburgh, PA) scintillation fluid and the radioactivity was determined by liquid scintillation counting.

Plasma Homocysteine Determination

Total plasma homocysteine concentrations were analyzed as described by Ubbink *et al.* (278). Briefly, plasma samples (300 μL) were incubated at 4°C for 30 minutes with 10 percent tributylphosphine in dimethylformamide (v/v). *N*-acetylcysteine (1 mM) was added to the plasma samples as an internal standard. Following termination of the reaction with ice-cold 10 percent trichloroacetic acid (w/v) containing 1 mM EDTA, samples were centrifuged at $1,000 \times g$ for 5 minutes. The supernatant fraction was removed and added to a solution containing 0.125 M borate buffer (pH 9.5), 1.55 M sodium hydroxide, and 0.1 percent (w/v) 4-fluoro-7-sulfobenzofurazan (ammonium salt). Homocysteine was quantified using HPLC in conjunction with fluorometric detection as described (198, 230).

MS and BHMT Activity Analysis

MS activity was analyzed using the method described by Keating *et al.* (134). The assay was initiated with 600 μg of protein added to a reaction mixture (200 μL) containing the following: 105 mM sodium phosphate buffer (pH 7.5), 0.12 μM cyanocobalamin, 36 mM dithiothreitol, 0.3 mM SAM, 8.4 mM β -mercaptoethanol, 8.25 mM DL-homocysteine, and 0.63 mM [*methyl*-¹⁴C]-THF (0.17 $\mu\text{Ci}/\mu\text{mol}$). Following incubation at 37°C for 1 hour, the reaction was terminated by adding ice-cold water. Samples were immediately transferred to AG 1-X8 (chloride form) resin columns and effluent fractions collected for liquid scintillation counting. The enzymatic activity of BHMT was determined as previously described (92) in duplicate using a reaction mixture containing 40 μg protein, 50 mM Tris-HCl (pH 7.5), 5 mM DL-homocysteine, and 2 mM betaine (0.05-0.1 μCi). Following a 1- to 2-hour incubation at 37°C, samples were chilled, ice-cold water added, and applied to Dowex 1-X4 columns. Eluted fractions were collected in scintillation vials and radioactivity was measured by liquid scintillation counting. One unit of BHMT activity was equivalent to the

production of 1 nmol of methionine per hour (211). For both assays, fresh homocysteine was prepared daily from the thiolactone derivative (92).

Determination of GNMT and BHMT mRNA abundance

Total mRNA was isolated from frozen liver using a commercial reagent kit (RNAeasy™, Qiagen) and quantified by UV detection. Northern blot analysis was performed as described previously (255). Liver RNA was resolved on 1.2 percent (w/v) agarose gels containing formaldehyde, transferred to Nytran Super Charge membranes (Schleicher & Schuell, Bioscience, Keene, NH) using a Turboblotter (Schleicher & Schuell), and RNAs were immobilized by UV-cross-linking. Membranes were prehybridized in ExpressHyb (BD Biosciences Clontech, Palo Alto, CA) and then hybridized with ³²P-labeled probes. The GNMT probe was a *Nde*I/ *Kpu*I restriction fragment from the 3' end of the rat GNMT in pEt-17b that was kindly provided by Dr. Zigmund A. Luka, Vanderbilt University Medical Center, Nashville, TN. The BHMT probe was a *Eco*RI restriction fragment from BHMT cDNA (nt 866-1317). DNA probes were labeled using a Ready-To-Go DNA labeling beads (Amersham Biosciences, Piscataway, NJ) and (α -³²P) dCTP (3000 Ci/mmol, PerkinElmer, Life & Analytical Sciences, Boston, MA). After hybridization, membranes were washed and evaluated by PhosphorImager analysis (Sunnyvale, CA) using ImageQuant software. To normalize RNA expression, membranes were stripped and rehybridized with labeled rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes (Ambion, Inc., Austin, TX).

Measurement of Hepatic Folate Concentrations

THF and 5-CH₃-THF were determined using HPLC and fluorometric detection according to Rebello (221) with some minor modifications (208). Briefly, frozen homogenates were thawed and placed in a boiling water bath for 60 minutes and following centrifugation at 20,000 × g for 10 minutes, rat serum conjugase was added to an aliquot of the resulting supernatant and incubated for 1 hour at 37 °C. Following activation of Sep-Pak NH₂ columns with acetonitrile and 16 mM sodium acetate buffer (pH 4.5), samples were applied and washed with acetate buffer and sodium phosphate (100 mM) containing 50 mM 2-mercaptoethanol. Folate coenzymes were separated on a phenyl Radial-Pak column (Waters Associates, Milford, MA) and quantified using fluorometric detection.

Statistical Analysis

The mean values of each treatment group were analyzed by a two-way ANOVA using SigmaStat software (SPSS, Chicago, IL) at a significance level of 5 percent and compared using Fisher's least significant difference procedure (254). Mean GNMT and BHMT mRNA values in control and diabetic rats were analyzed by a Student's *t*-test ($p \leq 0.05$).

Results

Confirmation of differential folate status and a diabetic state in rats. Neither STZ treatment nor dietary folate levels had a significant effect on the weight gain (**Table 4.2**). Blood glucose concentrations in STZ-treated rats were approximately 2-fold greater in all diet groups compared to their respective controls, regardless of dietary folate levels. Control rats fed the folate-deficient diet exhibited an 84 percent reduction in total hepatic folate coenzyme concentrations, whereas they were increased 56 percent by folate supplementation (FD, 1.18 ± 0.11 ; F, 7.37 ± 0.92 ; FS, 11.53 ± 0.94 nmol/g liver). Taken together, the data indicates that rats were in a moderate state of diabetes and altered folate status.

Folate-containing diets attenuated GNMT activity in diabetic rats, but was without effect on abundance. Mild folate deficiency in diabetic rats resulted in a 200 percent induction of GNMT activity as compared to non-diabetic controls (**Figure 4.2A**). However, this induction was significantly attenuated 64 and 74 percent in treatment groups fed the folate-adequate and folate-supplemented diets, respectively, compared to diabetic rats fed a folate-deficient diet. In contrast, GNMT abundance was elevated to the same extent in all diabetic rats regardless of dietary folate (**Figure 4.2B**). Taken together, these results indicate that adequate dietary and subsequently hepatic folate concentrations attenuated the diabetes-mediated increase in GNMT activity, likely by posttranslational control.

Hyperhomocysteinemia in folate-deficient rats was attenuated by streptozotocin (STZ)-induced diabetes. Folate-deficient animals exhibited a 4- to 5-fold increase in total plasma homocysteine concentrations compared to animals fed either the folate-adequate or folate-supplemented diet (**Figure 4.3**). However, homocysteine levels in folate-deficient diabetic rats were only 54 percent of that exhibited by non-diabetic rats. As expected, normal homocysteine concentrations were observed in folate-adequate and folate-supplemented

control rats. The lower mean homocysteine concentrations in diabetic rats provided an adequate or supplemented folate diet were similar.

Diabetes elevated the hepatic activity of PEMT and BHMT, whereas MS activity was diminished in diabetic rats. The enzymatic activity of PEMT was determined as it is a major user of methyl groups from SAM, thereby reflecting methyl group homeostasis, as well as a regulator of homocysteine balance. As shown in **Table 4.3**, the activity of SAM-dependent PEMT was increased ~2-fold in all diabetic rats, regardless of dietary folate. The enzymatic activity of the two hepatic enzymes required for folate-dependent and folate-independent remethylation of homocysteine, MS and BHMT, respectively, are also shown in Table 2. A diabetic state differentially altered the activity of these enzymes. For all of the folate diet groups, BHMT activity was increased approximately 1.5- to 2-fold compared to their respective non-diabetic controls. Conversely, STZ treatment reduced MS activity 23-46 percent compared to control values.

GNMT and BHMT mRNA abundance were increased in diabetic rats. The elevated activity of BHMT and GNMT in diabetic rats was reflected in the abundance of their respective mRNAs. GNMT and BHMT mRNA levels were elevated 6.5- and 1.9-fold in diabetic rats, respectively, compared to the non-diabetic animals (**Figure 4.4**).

Discussion

Understanding the nutritional and/or physiologic factors that modulate folate, homocysteine, choline, and methyl group metabolism is critical towards the prevention of numerous pathologies associated with perturbation of these pathways (132, 227, 240). We have shown previously (198) that a *type I* diabetic state results in modulation of methyl group metabolism by increasing the enzymatic activity and protein abundance of GNMT, a key hepatic protein in the regulation of methyl group supply from the folate-dependent one carbon pool and its utilization for SAM-dependent transmethylation reactions. Because induction of GNMT function would be expected to result in a loss of methyl groups as well as contribute to homocysteine production, developing intervention strategies directed at restoring normal GNMT function is important. Thus, the aim of this research was to determine the impact varying degrees of folate status has on diabetes and methyl group metabolism, particularly as the folate coenzyme 5-CH₃-THF allosterically inhibits GNMT

activity (291, 292). Moreover, we also examined the effects of a diabetic state on additional aspects of SAM-dependent metabolism of methyl groups and choline, namely phospholipid methylation.

A lack of dietary folate clearly resulted in the highest activity of GNMT in diabetic rats without altering the abundance of the protein. Because GNMT was attenuated in diets containing adequate or supplemental folate, together these results would indicate that modulation of GNMT activity, as expected, was at a posttranslational level as a result of diminished 5-CH₃-THF concentrations. We have found similar results for retinoic acid-mediated induction of GNMT in rats fed a folate-deficient diet (VE Knoblock and KL Schalinske, unpublished data). Although these results would suggest that adequate folate status has a positive effect under diabetic conditions, supplemental folate did not confer any added benefit. Providing folate at levels ~20-fold greater than our studies here, others have shown that folate supplementation by injection was an effective means to reduce embryo malformations in diabetic rats (299). Thus, it will be important in future research to determine if a greater reduction in GNMT activity can be achieved with higher doses of folate, route of supplementation, and/or longer treatment times.

The marked hyperhomocysteinemia exhibited in the folate-deficient rats was surprising, given that the degree of folate deficiency would appear to be moderate. This assessment is based on the findings that no changes were seen in growth and an antibiotic was not added to the drinking water to eliminate bacterial folate production. A similar 4-wk study design with the inclusion of an antibiotic resulted in only a 2-fold increase in plasma homocysteine concentrations (117). However, we found that hepatic folate concentrations were only 16 percent of that observed for folate adequate rats. Hyperhomocysteinemia in folate-deficient rats was significantly attenuated as a result of diabetes, likely the result of an increase in BHMT activity. Our previous work and others have shown that a diabetic condition can also reduce circulating homocysteine levels as a result of increased homocysteine catabolism via CBS (123, 198, 219). In contrast to previous studies (123, 198, 219), hypohomocysteinemia was not statistically evident in folate-adequate or folate-supplemented rats receiving an amino acid-defined diet.

We have extended our previous work by demonstrating that the induction of GNMT and BHMT is reflected in the abundance of their respective mRNAs. It is not known what signal results in increased mRNA of these proteins, either by a transcriptional mechanism or through mRNA stability. Previous studies have shown that various hormones (e.g., dexamethasone) and relevant metabolites (e.g., methionine, SAM) have the ability to directly regulate the expression of these proteins (46, 228, 230). Ultimately, the induction of GNMT and BHMT would be expected to have an impact on other SAM-dependent methyltransferases and choline metabolism, respectively.

For humans, there are numerous reports in the literature that have found both *type 1* and *type 2* diabetes are associated with hyperhomocysteinemia, thereby establishing a potential link to increased cardiovascular disease risk (114, 183, 216, 224). The hypohomocysteinemia that has been reported by our laboratory and others (123, 198, 219) is likely due to the role of the kidneys in homocysteine metabolism. The progression from a hypo- to a hyperhomocysteinemic state may be the result of renal dysfunction (216), as the kidneys play a significant role in the metabolism of homocysteine (26, 80). Taken together with the findings reported here, it is clear that future research will need to be directed at understanding the tissue-specific temporal changes in methyl group and homocysteine metabolism as a function of diabetes progression. This knowledge will be vital in the development of dietary and/or therapeutic intervention strategies aimed at preventing disruption of these pathways by a diabetic condition.

A novel finding reported here is the up-regulation of the enzymatic activity of PEMT by diabetes. There are numerous studies that have examined the impact of a diabetic state on the regulation of PEMT activity and PC synthesis; however, the reports are conflicting and appear to vary depending on the animal model, method for inducing diabetes, and the type of tissues examined (36, 45, 270, 307). The action of PEMT constitutes a major route for utilization of the labile methyl groups from SAM (126) and thus would be expected to provide some index of methyl group availability. Moreover, PEMT is responsible for ~30 percent of PC production with the remainder being derived directly from choline pools. Our observed increase in PEMT activity may reflect a shift from using PE, rather than choline

directly, for PC synthesis owing to the loss of choline, as betaine, for BHMT and homocysteine remethylation.

PEMT expression and function has been reported to significantly influence homocysteine production and secretion (200, 249). Moreover, it has been proposed to represent a more significant use for SAM-derived methyl groups, as opposed to SAM-dependent synthesis of creatine (126). These previous studies reported that homocysteine secretion positively associated with inducing expression of PEMT in hepatoma cells, and a complete lack of PEMT expression resulted in low circulating levels of homocysteine in PEMT knockout mice (200, 249). Similarly, a 2-fold increase in PEMT activity, similar to the elevation we have observed in diabetic rats, in CTP:phosphocholine cytidylyltransferase- α knockout mice also resulted in an increase in homocysteine production and secretion (126). Thus, the absence of PEMT expression or increasing its expression in cells that have low levels of expression appears to regulate homocysteine balance. However, our studies indicate that an increase in hepatic PEMT activity *in vivo*, as the result of a diabetic state, does not have a concomitant alteration in circulating homocysteine levels. It is likely that the diabetes-mediated increase in PEMT, as well as GNMT, does result in an increase in homocysteine production, but stimulation of BHMT expression is a compensatory mechanism to prevent homocysteine pools from accumulating. Moreover, irreversible catabolism of homocysteine by the transsulfuration pathway and the initial action of CBS has been shown to be activated by diabetes and glucocorticoids (123, 219).

The collective induction of PEMT, GNMT, and BHMT, combined with diminished MS activity, indicates that diabetes is characterized by a deficiency of methyl groups and an increased requirement for choline. This is supported by our preliminary studies that found the hepatic synthesis of creatine was significantly decreased ~50 percent in diabetic rats (CS Hartz and KL Schalinske, unpublished observations), indicating that the induction of GNMT and PEMT results in a deficiency of adequate methyl groups for other SAM-dependent transmethylation reactions. To that extent, we have found that diabetes is characterized by hypomethylation of DNA in rat liver (303). The utilization of PC for bile secretion is greatly enhanced in the diabetic state (288) and PC requirements correlate to PEMT activity (122). Studies that focus on the measurement of metabolic flux through these pathways, similar to

what has been recently reported (62, 65), needs to be performed to definitively evaluate the effect of diabetes. Recently, a kinetic study with human subjects demonstrated that transmethylation, homocysteine transsulfuration, and clearance of homocysteine were significantly reduced in type 2 diabetes with nephropathy (271).

It is also clear from our findings that the observations reported here are specific for a diabetic state and not the result of using a chemically-induced model. This is supported by the fact that we, and others, have found similar changes using an either alloxan-mediated model, or treating rats and cultured hepatoma cells with dexamethasone (230, 307, 310). Most recently, we have also demonstrated that treatment of STZ-diabetic rats with insulin prevented the induction of GNMT and PEMT, and restored circulating homocysteine concentrations to normal levels (199) (Chapter 3).

In summary, we have shown that *type 1* diabetes results in the metabolic disruption of methyl groups, choline, and homocysteine, and folate status has an impact on these findings. This may have significant implications with respect to the nutritional needs of patients with *type 1* diabetes. Importantly, many of these metabolic findings with respect to *type 1* diabetes have also been recently noted in a *type 2* diabetes model (299). For humans with diabetes, additional factors that will clearly play a significant role in modulating methyl group and homocysteine metabolism include other moderate B-vitamin (e.g., B₁₂ and B₆) deficiencies and/or expression of polymorphic enzymes, such as MTHFR.

Footnotes

¹ Nieman KM³, Hartz CS³, Szegedi SS⁴, Garrow TA⁴, Sparks JD⁵, Schalinske KL³. Folate status modulates the induction of hepatic glycine *N*-methyltransferase and homocysteine metabolism in diabetic rats. *Am J Physiol Endocrinol Metab* 291:1235-1242, 2006. This work was supported in part by: the American Diabetes Association (K.L.S.); the Cancer Research and Prevention Foundation (K.L.S.); and National Institutes of Health Grants DK52501 (T.A.G.), HL65398 (J.D.S.), and DK50376 (J.D.S.).

² Nieman KM³, Schalinske KL³. Folate status modulates the induction of glycine *N*-methyltransferase, whereas hyperhomocysteinemia due to folate deficiency was attenuated in diabetic rats. *FASEB J* 19(4): A50, 2005 (abstr.).

³ Graduate student(s) and associate professor (K.L.S), Interdepartmental Graduate Program in Nutritional Sciences and the Department of Food Science and Human Nutrition, Iowa State University, Ames, Iowa.

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⁵ Associate professor, Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, New York

Tables

Table 4.1

Standard Amino Acid Defined Diet (F) ¹

Diet Constituent	g/kg	Diet Constituent	g/kg
L-Alanine	3.5	Corn Starch	150.0
L-Arginine	12.1	Maltodextrin	150.0
L-Asparagine	6.0	Cellulose	30.0
L-Aspartic Acid	3.5	Soybean Oil	80.0
L-Cystine	3.5	Mineral Mix ²	35.0
L-Glutamic Acid	40.0	Calcium Phosphate Monobasic	8.2
Glycine	23.3	Choline Bitartrate	2.5
L-Histidine	4.5	Niacin ³	0.03
L-Isoleucine	8.2	Calcium Pantothenate ³	0.016
L-Leucine	11.1	Pyridoxine HCl ³	0.007
L-Lysine HCl	18.0	Thiamin HCl ³	0.006
L-Methionine	8.2	Riboflavin ³	0.006
L-Phenylalanine	7.5	Folic Acid ^{3,4}	0.002
L-Proline	3.5	Biotin ³	0.0002
L-Serine	3.5	Vitamin B ₁₂ (0.1% in mannitol) ³	0.025
L-Threonine	8.2	DL- α Tocopheryl Acetate (500 IU/g) ³	0.15
L-Tryptophan	1.8	Dry Vitamin A Palmitate (500,000 U/g) ³	0.008
L-Tyrosine	5.0	Vitamin D ₃ , cholecalciferol (500,000 U/g) ³	0.002
L-Valine	8.2	Vitamin K, phylloquinone ³	0.0008
Sucrose	364.429	TBHQ (antioxidant)	0.02

¹The folate-adequate diet (F) was a custom research diet developed by Harlan Teklad (TD.03333, Madison, WI).

²Mineral Mix, AIN-93M-MX (TD 94049).

³Diet constituents are part of vitamin mix AIN-93-VX (TD 94047).

⁴Folate-deficient (FD, TD.03428) and folate-supplemented (FS, TD.03429) diets were modified by excluding folate and adding 6 ppm folate, respectively.

Table 4.2

Cumulative weight gain, blood glucose concentrations, and hepatic folate concentrations in rats treated with streptozotocin (STZ) and fed either 0 (FD), 2 (F), or 8 (FS) ppm dietary folate¹

	Weight Gain (g)	Plasma glucose (mM)
FD	143 ± 12 ^a	10.4 ± 0.7 ^a
FD + STZ	122 ± 7 ^a	21.5 ± 2.7 ^b
F	144 ± 19 ^a	11.5 ± 0.8 ^a
F + STZ	137 ± 5 ^a	20.9 ± 2.0 ^b
FS	131 ± 13 ^a	10.1 ± 1.2 ^a
FS + STZ	128 ± 3 ^a	21.8 ± 1.5 ^b

¹Values are means ± SE; n = 6. Different letters within a column indicates a significant difference, $p \leq 0.05$.

Table 4.3

Hepatic activity of the SAM-dependent methyltransferase PEMT and homocysteine remethylation enzymes BHMT and MS in rats treated with streptozotocin (STZ) and fed either 0 (FD), 2 (F), or 8 (FS) ppm dietary folate¹

	PEMT* (pmol/min·mg protein)	BHMT (U/mg protein)	MS (pmol/min·mg protein)
FD	29.1 ± 1.2 ^a	63 ± 6 ^a	54 ± 5 ^a
FD + STZ	55.9 ± 11.5 ^b	114 ± 16 ^c	29 ± 3 ^c
F	31.5 ± 2.3 ^a	58 ± 6 ^a	47 ± 6 ^{a,b}
F + STZ	58.9 ± 6.4 ^b	91 ± 14 ^b	36 ± 6 ^{b,c}
FS	31.6 ± 2.7 ^a	51 ± 4 ^a	50 ± 6 ^{a,b}
FS + STZ	69.1 ± 6.2 ^b	93 ± 6 ^{b,c}	31 ± 3 ^c

¹Values are means ± SE; n = 6. Different letter superscripts within a column indicate a significant difference, $p \leq 0.05$. * Abbreviations: BHMT, betaine homocysteine S-methyltransferase; MS, methionine synthase; PEMT, phosphatidylethanolamine N-methyltransferase.

Figure Legends

Figure 4.1

Folate, homocysteine, choline and methyl group metabolism. The primary methyl donor *S*-adenosylmethionine (SAM) provides methyl groups for numerous of methyltransferase reactions including: the conversion of glycine to sarcosine by glycine *N*-methyltransferase (GNMT), the synthesis of phosphatidylcholine (PC) from phosphatidylethanolamine (PE) by phosphatidylethanolamine *N*-methyltransferase (PEMT), and creatine phosphate production by the action of guanidoacetate methyltransferase (GAMT). *S*-adenosylhomocysteine (SAH) results following methyl group donation and is subsequently hydrolyzed to homocysteine by SAH hydrolase (SAHH). Homocysteine can either be further catabolized by the transsulfuration pathway to cysteine, by the activity of cystathionine β -synthase (CBS) and γ -cystathionase, or remethylated back to methionine. Homocysteine remethylation occurs by the folate-independent pathway and the activity of betaine-homocysteine-*S*-methyltransferase (BHMT), or through the folate-dependent activity of methionine synthase (MS), a B₁₂-dependent enzyme that reduces 5-methyltetrahydrofolate (5-CH₃THF) to tetrahydrofolate (THF). The majority of one-carbon groups for the folate-dependent one-carbon pool originate from serine and serine hydroxymethyltransferase (SHMT) to generate 5,10-CH₂-THF, which can then undergo irreversible reduction to 5-CH₃-THF by the action of 5,10-CH₂-THF reductase (MTHFR).

Figure 4.2

Induction of hepatic glycine *N*-methyltransferase (GNMT) in streptozotocin (STZ)-mediated diabetic rats. Rats were fed a diet consisting of 0 ppm (folate-deficient, FD), 2 ppm (folate-adequate, F) or 8 mg folate/ kg diet (folate-supplemented, FS) for a period of 35 days. A single dose of streptozotocin (STZ, 60 mg/kg body weight) was administered on day 30. Liver samples were collected and the activity and abundance of GNMT was determined as described under “Materials and Methods”. **A:** GNMT activity in diabetic and non-diabetic rats fed the various folate diets. Data are expressed as means \pm S.E. ($n = 6$) and bars denoted with different letters are significantly different ($p \leq 0.05$). **B:** GNMT abundance in diabetic and non-diabetic rats fed the various folate diets. A monoclonal GNMT antibody (24) was used for Western blot analysis, and a representative immunoblot is shown. Data are

expressed as means \pm SE (n = 6) and displayed as a percent of the mean of the control group. Bars denoted with different letters are significantly different ($p \leq 0.05$).

Figure 4.3

Hyperhomocysteinemia in folate-deficient rats was attenuated by streptozotocin (STZ)-mediated diabetes. Plasma samples from the same rats as described for Figure 4.2 were obtained for the determination of total homocysteine concentrations by HPLC and fluorometric detection as described under “Materials and Methods”. Data are expressed as means \pm SE (n = 6) and bars denoted with different letters are significantly different ($p \leq 0.05$).

Figure 4.4

GNMT and BHMT mRNA abundance were increased in streptozotocin (STZ)-mediated diabetic rats. Liver samples from the same rats as described for Figure 4.2 were collected for the isolation of total RNA. The abundance of GNMT and BHMT mRNA was determined by Northern blot analysis as described under “Materials and Methods”. Data are expressed as means \pm SE (n = 3-9). * $P \leq 0.05$ vs. non-diabetic values. GNMT and BHMT mRNA abundance values were normalized to GAPDH mRNA abundance for each sample.

Figures

Figure 4.1

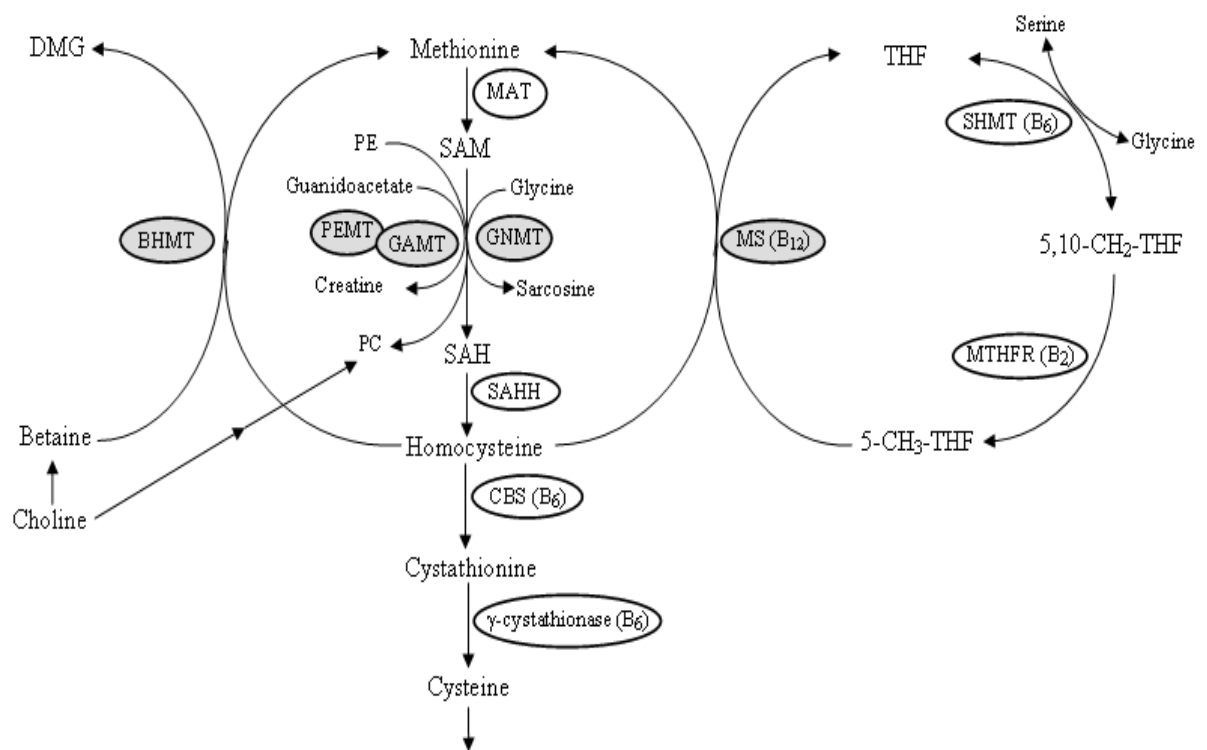


Figure 4.2A

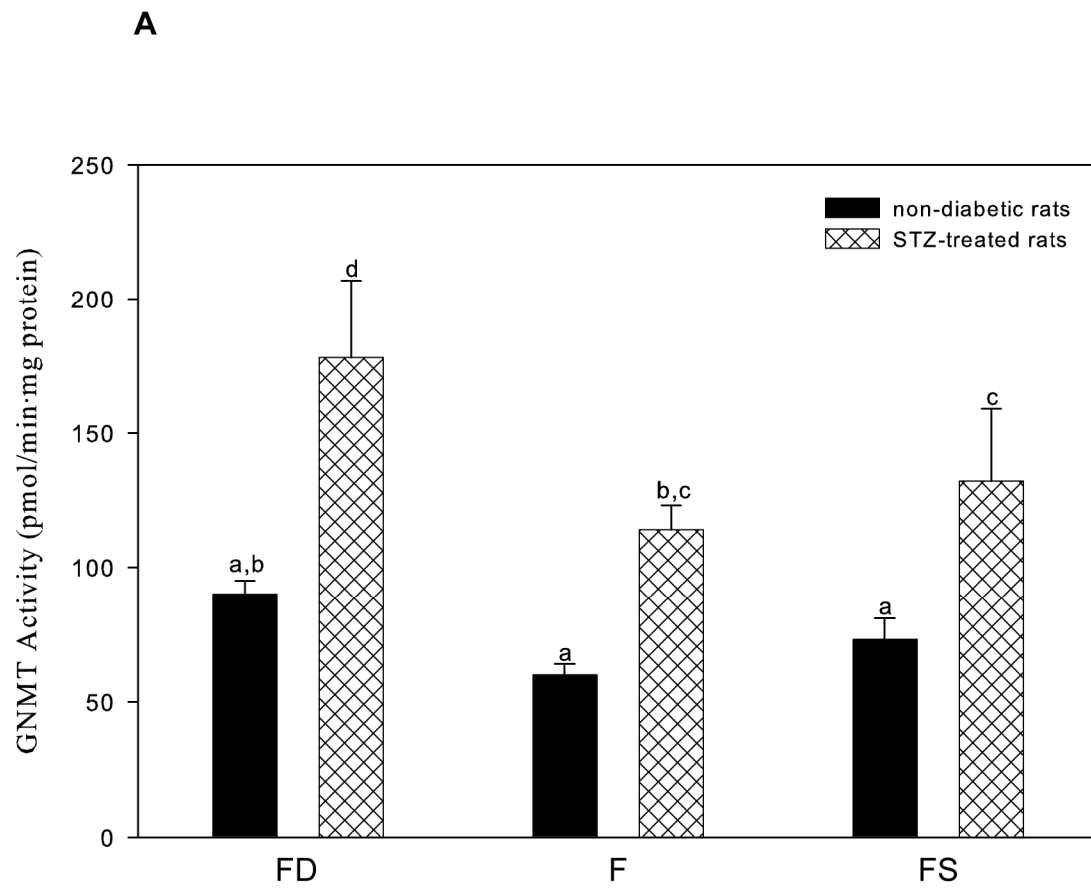


Figure 4.2B

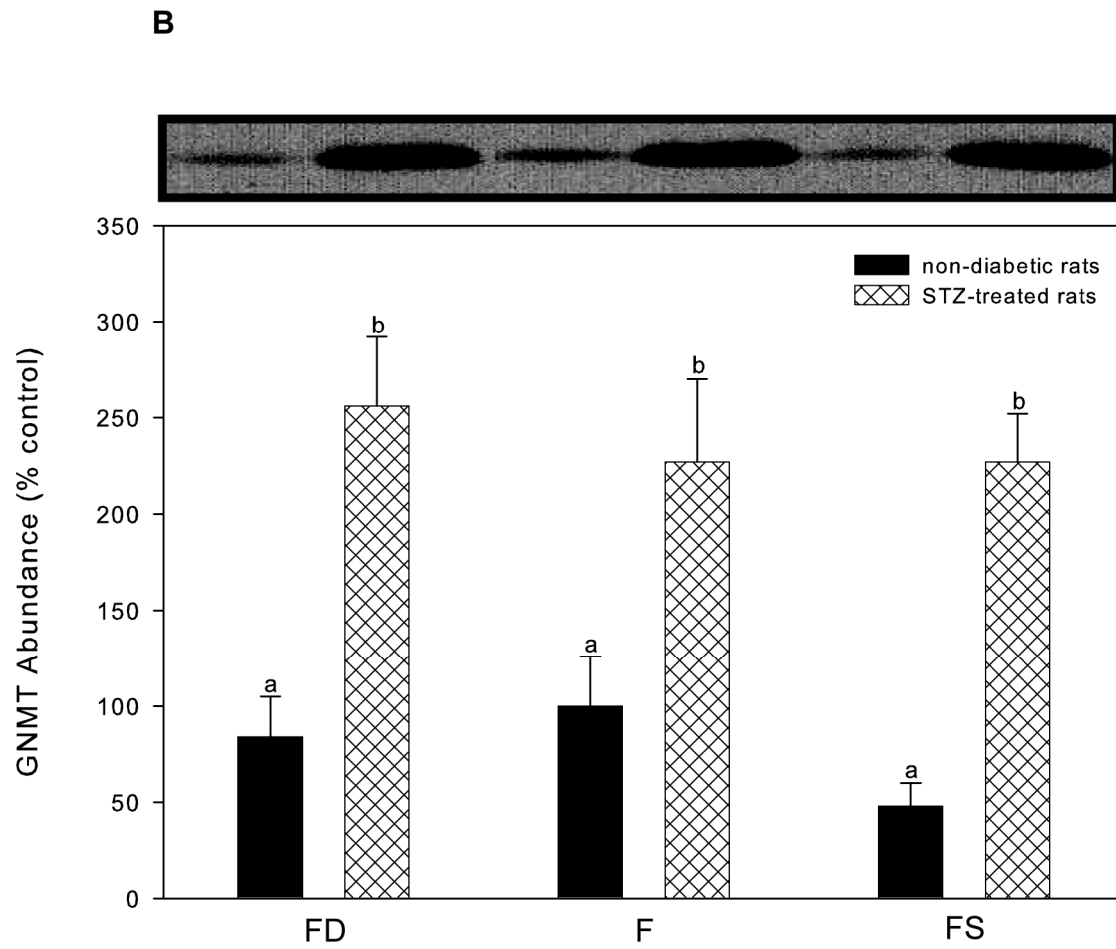


Figure 4.3

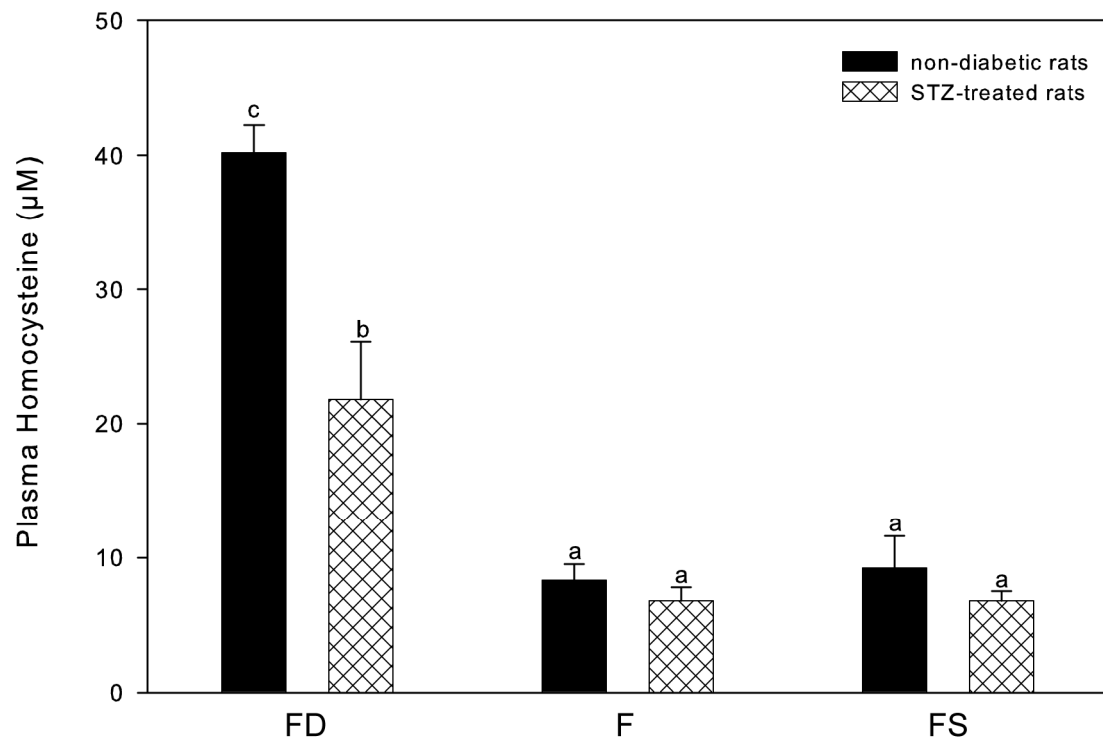
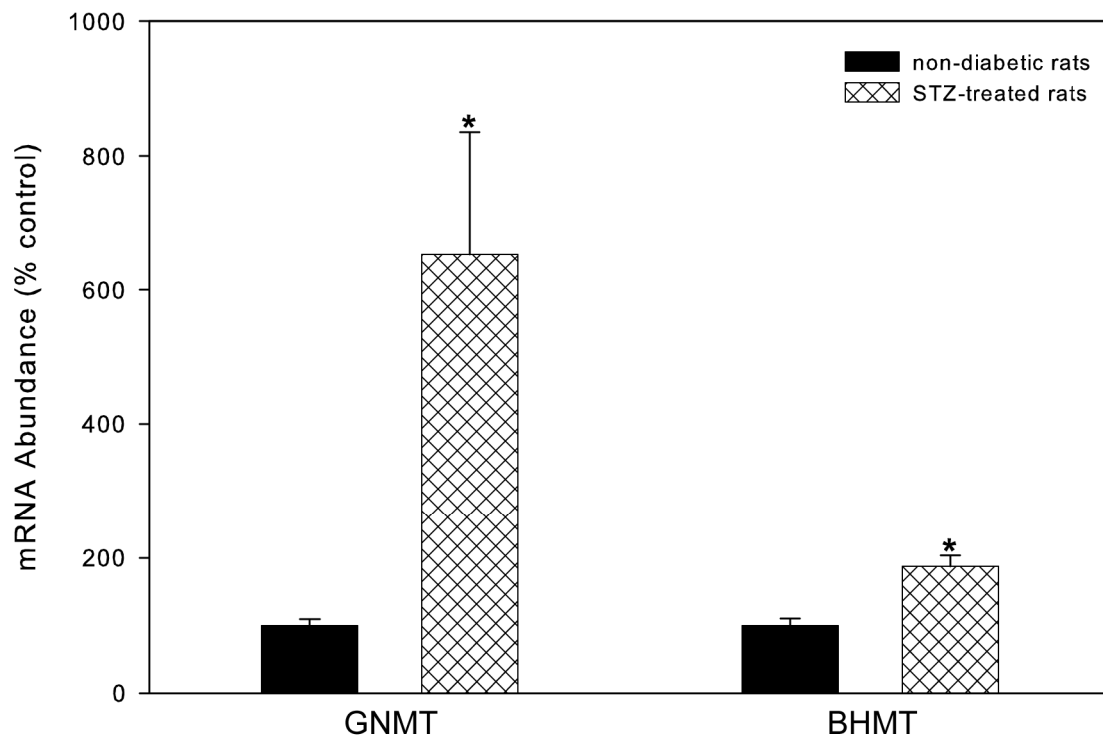


Figure 4.4

CHAPTER 5: VITAMIN D SUPPLEMENTATION MAINTAINS NORMAL HOMOCYSTEINE METABOLISM IN DIABETIC RATS

Modified from an abstract published in the *Federation of American Societies for
Experimental Biology Journal*¹

Abstract

A diabetic state is known to disrupt both methyl group and homocysteine metabolism. Recent studies indicate acute diabetes leads to both an induction of glycine-*N*-methyltransferase (GNMT), a key regulatory enzyme in methyl group metabolism, and hypohomocysteinemia. However, as kidney function deteriorates hyperhomocysteinemia results in chronic diabetes, a link to complications such as cardiovascular disease. A number of nutrients have been identified as modifiers of diabetic pathogenesis including vitamin D. Studies have reported increased insulin sensitivity and a reduction in blood glucose with vitamin D supplementation. Supplementation with vitamin D has been shown to prevent development of type 1 diabetes in genetic mouse model, whereas vitamin D deficiency has been shown to increase the risk for diabetic pathogenesis. Therefore, this study was conducted to determine if vitamin D supplementation restores normal methyl group and homocysteine metabolism in diabetic rats. Rats were randomly assigned to one of four groups: control, streptozotocin-induced diabetic (STZ, 60 mg/kg BW), vitamin D (D, calcitriol, 500 ng/kg BW), or both (D+STZ). Vitamin D supplementation prevented hypohomocysteinemia in diabetic rats, which is likely due to an induction of GNMT. It did not prevent other diabetes-mediated alterations in homocysteine remethylation or transsulfuration. Taken together, it appears that vitamin D supplementation may be of benefit by maintaining normal homocysteine metabolism when disrupted by a diabetic state, resulting from increased production of homocysteine.

Introduction

The metabolism of folate, methyl groups, and homocysteine are key interrelated pathways involved in optimal health and disease prevention. Thus, disruption of these metabolic pathways risk development of a number of pathologies including cancer, vascular disease,

and neural tube defects (132, 227, 240). Methyl groups are most often provided in the diet (*i.e.* methionine and choline) or endogenously from the folate-dependent one-carbon pool. In the form of methionine, adenylation activates this amino acid forming *S*-adenosylmethionine (SAM), the primary methyl group donor in over 50 known mammalian transmethylation reactions (194, 289) (**Figure 5.1**). *S*-adenosylhomocysteine (SAH) results following methyl group donation in biosynthetic- and modification-type reactions, such as nucleic acids and specialized proteins ($X \rightarrow X-CH_3$). The production of phosphatidylcholine (PC) from phosphatidylethanolamine (PE) constitutes a major consumption of SAM-derived methyl groups through the activity of phosphatidylethanolamine *N*-methyltransferase (PEMT) activity (256). Thus PEMT is considered to be a major contributor to homocysteine production. Studies indicate that PEMT is regulated based on the need for PC and very low density lipoprotein secretion (122, 201) as well as homocysteine production (249). SAH is a potent inhibitor of SAM-dependent transmethylation reactions (40) making it vital to optimize the SAM:SAH ratio. This regulation is accomplished by the abundant tissue-specific cytosolic protein GNMT, in the conversion of glycine to sarcosine (135, 289, 310). SAH is hydrolyzed to homocysteine by SAH hydrolase (SAHH) (40). At this point homocysteine has two potential fates, remethylation to methionine or transsulfuration to cysteine.

SAM-dependent DNA methylation is catalyzed by a family of DNA methyltransferases (DNMTs) (156), which transfer a methyl group to cytosine in CpG dinucleotides. Epigenetic modification of DNA by methylation also requires methyl groups from SAM; as a result methylation status is an indicator of methyl group supply. DNA methylation is a critical tool in epigenetic modification, regulating the tissue-specific expression of certain genes, and ultimately genome function (222). CpG islands are usually clustered in gene promoter regions, thus methylation often results in regulation of DNA expression, typically repression (130). Aberrant DNA methylation is also associated with a number of pathologies. Preneoplastic and tumor DNA is characterized by global hypomethylation, regional hypermethylation, and increased activity of DNMTs (20, 129, 151). Development of atherosclerotic lesions has also been associated with changes in DNA methylation (73, 110).

Remethylation of homocysteine to methionine occurs by two means, folate-independent or folate-dependent pathways, each equally contributing to the remethylation of homocysteine (85). A methyl group is donated by 5-methyl tetrahydrofolate (5-CH₃THF) in the folate-dependent remethylation of homocysteine to methionine. This reaction, catalyzed by the B₁₂-dependent enzyme methionine synthase (MS), results in tetrahydrofolate (THF) (248). Folate-independent remethylation of homocysteine occurs through the methyl donor betaine, which is formed following the oxidation of choline. This reaction is catalyzed by betaine homocysteine S-methyltransferase (BHMT), forming dimethylglycine and methionine (81, 92).

Homocysteine can also be irreversibly catabolized to cysteine through the oxidative transsulfuration pathway. Transsulfuration is accomplished by the activity of two B₆-dependent enzymes. Initially, cystathionine β-synthase (CBS) condenses homocysteine with serine forming cystathionine. Finally cystathionine is hydrolyzed to cysteine and α-ketobutyrate by γ-cystathionase (116, 147). Ultimately cysteine can be used to synthesize useful compounds such as glutathione, taurine, and pyruvate (260, 262). Thus measuring any of these metabolites would indicate relative homocysteine catabolism. Increased plasma homocysteine (271), is recognized as an independent risk factor for the development of vascular disease (31, 55, 132).

A number of factors (*i.e.* nutritional, hormonal, pharmacological, pathological, environmental, and genetics) have been identified as having the ability to influence homocysteine and methyl group metabolism. A diabetic state, characterized by a reduction of insulin levels and an elevation in circulating counter-regulatory hormones to insulin (*i.e.* glucagon and glucocorticoids) (304), has been indentified in the disruption of homocysteine and methyl group metabolism. In both type 1 and 2 diabetes without renal complications hypohomocysteinemia has been reported in humans and rats (8, 123, 198, 224, 299). Similar findings have been reported in animals treated with glucocorticoids and glucagon (124, 219, 230). Hypohomocysteinemia likely results from increased transsulfuration as evidenced by increased CBS activity, protein, and mRNA levels as well as γ-cystathionase activity (198, 219, 299). Folate-independent remethylation via BHMT may also play a role in reducing homocysteine, as its activity and mRNA levels have also been reportedly increased in acute

diabetes (197, 198, 220, 299). As reported in the following chapter and additional studies, insulin prevented hypohomocysteinemia in the STZ-induced type 1 diabetic rat (123, 199, 219). However, in type 1 and 2 diabetics, as renal function declines homocysteine levels rise (53, 118, 216). The hyperhomocysteinemic state that results may be attributed to decreased clearance of the sulfur-containing amino acid (271).

A growing collection of evidence indicates a relationship between vitamin D and diabetes. In NOD mice, treatment with the metabolically active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃ or calcitriol), prevented the onset of type 1 diabetes (175, 315). It has also been suggested that increasing vitamin D intake as early as infancy, may decrease the risk of the child developing type 1 diabetes later in life (121). Moreover vitamin D deficiency seemingly accelerates diabetes onset in NOD mice (96). In type 2 diabetic humans and animals, vitamin D deficiency has been reported to inhibit insulin secretion and impair glucose tolerance (54, 93, 202). Furthermore, vitamin D repletion seems to ameliorate the noted abnormalities in insulin secretion and glucose tolerance (131, 203) and glucose concentrations in type 2 diabetic rats were reduced by 40% with vitamin D₃ supplementation (69). Epidemiological evidence has also indicated that serum 25-hydroxyvitamin D₃ levels, the primary circulating form of vitamin D, are inversely correlated with the development of type 2 diabetes and insulin resistance (243).

Clearly, vitamin D status plays a role in the development and progression of both type 1 and 2 diabetes. Interestingly, as mentioned above, vitamin D promotes insulin secretion, which is also a known regulator of homocysteine metabolism. Thus, this study was aimed at determining if calcitriol supplementation could prevent diabetes-altered methyl group and homocysteine metabolism.

Materials & Methods

Chemicals

Reagents used in the research methods were obtained from the following sources: S-adenosyl-L-[*methyl*-³H]methionine, PerkinElmer Life Sciences; chemiluminescent Western blotting detection reagents, GE Healthcare; streptozotocin and S-adenosyl-L-methionine, Sigma-Aldrich; goat anti-rabbit IgG horseradish peroxidase, Southern Biotechnology; calcitriol, Biomol, Inc.; HpaII endonuclease, New England Biolabs Inc.; [³H]-dCTP, NEN

Life Science Products; GNMT polyclonal antibody and goat anti-chicken IgY horseradish peroxidase, Aves Labs, Inc. Jan Kraus, University of Colorado Health Sciences Center, generously provided the CBS polyclonal antibody. All other chemicals were of analytical grade.

Animals

Experiments involving animals were approved by and conducted in accordance with the Iowa State University Laboratory Animal Resources guidelines. Male Sprague-Dawley rats (125-149 grams) were obtained from Harlan Sprague-Dawley and housed in separate cages with a 12-hour light: dark cycle. Animals were allowed food and water *ad libitum*. Rats were acclimated to their diet (230) for 11 days and then randomly assigned to one of four treatment groups: control, diabetic (STZ), vitamin D-supplemented (D), and vitamin D-supplemented diabetic (D+STZ). Rats received an oral dose of 1, 25(OH)₂D₃ (500 ng/kg body weight) or the vehicle corn oil, beginning on day 12 of experiment and every other day until the end of the experiment. On day 21, the animals received a single intraperitoneal injection of either STZ (60 mg/kg body weight) or the vehicle (10 mM citrate buffer, pH 4.5). Animals were anesthetized on day 27 with a mixture of ketamine:xylazine (90:10 mg/kg body weight) and heparinized whole blood was collected by cardiac puncture. An aliquot was removed for the determination of blood glucose concentrations using a commercial kit (Sigma-Aldrich). The remaining whole blood was centrifuged at $5,000 \times g$ for 5 minutes and the plasma was removed and stored at -20°C for subsequent determination of homocysteine concentrations. Immediately following blood collection, portions of the liver were removed and homogenized in ice-cold buffer containing 10 mM sodium phosphate (pH 7.0), 0.25 M sucrose, 1 mM EDTA, 1 mM sodium azide, and 0.1 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at $20,000 \times g$ for 30 minutes and an aliquot of the supernatant was stored at -70°C for the subsequent determination of GNMT activity and abundance, MS activity, BHMT activity, and CBS abundance. An additional aliquot was removed and centrifuged at $100,000 \times g$. The pellet was collected for analysis of PEMT activity, resuspended in 0.25 M sucrose buffer, and stored at -70°C . Additional portions of the liver were collected and snap-frozen in liquid nitrogen for DNA isolation and methylation analysis. Total soluble protein concentration of the cytosolic and

microsomal fractions were determined using a commercial kit (Coomassie Plus, Pierce) based on the Bradford method (32) and bovine serum albumin as the standard.

Plasma Glutathione and Homocysteine Determination

Total plasma homocysteine and glutathione concentrations were analyzed as described by Ubbink *et al.* (278). Briefly, plasma thiol samples (300 μ L) were derivatized with 10% (v/v) tributylphosphine in dimethylformamide and incubated at 4°C for 30 minutes. *N*-acetylcysteine (1 mM) was also added to each sample as an internal standard. Following termination of the reaction with ice-cold 10% (w/v) trichloroacetic acid containing 1 mM EDTA, samples were centrifuged at $1,000 \times g$ for 5 minutes. The supernatant was removed and added to a solution containing 0.125 M borate buffer (pH 9.5), 1.55 M sodium hydroxide, and 0.1% (w/v) 4-fluoro-7-sulfobenzofurazan (ammonium salt). HPLC was used with fluorometric detection to quantify homocysteine by injecting the derivatized samples on to a μ -Bondapak C₁₈ Radial-Pak column (Waters Associates) equilibrated with 4% (v/v) acetonitrile in 0.1 M monobasic potassium phosphate buffer (pH 2.1) (198, 230).

GNMT Activity Determination

The enzymatic activity of GNMT was measured based on the method of Cook and Wagner (60). The assay was performed in triplicate using 250 μ g 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-³H]methionine. Samples were then incubated for 30 minutes at 25°C. Following incubation, 10% (w/v) trichloroacetic acid in 0.1 M acetic acid was added to stop the reaction, followed by activated charcoal (78 mg/mL) to absorb the unreacted SAM. Samples were centrifuged at $14,000 \times g$ for 5 minutes and 200 μ L of the supernatant was removed for liquid scintillation counting.

CBS and GNMT Abundance Determination

The abundance of CBS and GNMT were determined using immunoblotting methods as described (230). A 15% (w/v) SDS-polyacrylamide gel was polymerized and utilized to quantify the 32-kDa subunit of GNMT and the 63-kDa subunit of CBS using electrophoresis. Proteins were then transferred to nitrocellulose paper and incubated with a 1:50,000 dilution of the primary CBS antibody (rabbit) and a 1:5000 dilution of the primary GNMT antibody (chicken), overnight at 4°C. The next day the membrane was incubated with a 1:5000

dilution of either a goat anti-rabbit IgG horseradish peroxidase secondary antibody or a goat anti-chicken IgY horseradish peroxidase secondary antibody for one hour at room temperature. Proteins were detected using chemiluminescence and multiple exposures to Kodak X-Omat AR film. Band density was assessed using Quantity one[®] software (Biorad).

BHMT and MS Activity Analysis

BHMT activity was measured as previously described by Garrow (92). The standard assay mixture contained 5 mM DL-homocysteine, 2 mM betaine (0.1 μ Ci), and 40 μ g total protein. BHMT activity is expressed as units/mg protein, where 1 unit is equivalent to 1 nmol of methionine formed per hour. The activity of MS was determined as previously described (134). The assay reaction mixture containing 500 mM sodium phosphate buffer (pH 7.5), 1.3 μ M cyanocobalamin, 1M dithiothreitol, 10 mM SAM, 82.4 mM β -mercaptoethanol, 100 mM homocysteine, and 15 mM [*methyl*-¹⁴C]-THF (0.17 μ Ci/ μ mol), was added to liver supernatants and incubated for one hour at 37°C. Ice-cold water was added to stop the reaction and the assay mixture was immediately applied to an AG 1-X8 (chloride form) resin column. Effluent fractions (3 mL deionized water) were collected for subsequent liquid scintillation counting. Homocysteine was prepared fresh daily from a thiolactone derivative for both activity assays.

Determination of PEMT Activity

PEMT activity was determined by measuring the incorporation of radiolabeled methyl groups from S-adenosyl-L-[methyl-³H]methionine into phospholipids by the methods of Duce *et al.* (74). The reaction mixture contained 10 mM HEPES (pH 7.3), 4 mM dithiothreitol, 5 mM MgCl₂, 0.1 mM SAM, 2 μ Ci S-adenosyl-L-[methyl-³H]methionine, and 600 μ g protein in a final volume of 550 μ L. The reaction was initiated by adding 600 μ g microsomal protein (10 μ g/ μ L) to the assay mixture and incubated in a 37°C water bath for 15 minutes. The reaction was terminated by transferring 100 μ L 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 with 0.5 M KCl in 50% (v/v) methanol, transferred to glass scintillation vials, and allowed to dry at room temperature overnight. The following day, 5 mL scintillation fluid was added to each vial and the samples were then subjected to liquid scintillation counting.

Determination of Global DNA Methylation

Global hepatic DNA methylation was determined using an assay that measures *in vitro* incorporation of methyl groups into global DNA as described by Pogribny *et al.* (215), with minor modifications. DNA was purified from liver samples (186) using a commercial kit (Promega). The DNA (1.25 µg) was then digested using HpaII endonuclease (New England Biolabs, Inc.). The DNA extension assay mixture consisted of 1.0 µg digested DNA, 1X PCR Buffer II, 1mM magnesium chloride, 0.5 U AmpliTaq DNA polymerase (Applied Biosystems), and 9.3 µM [³H]-dCTP (NEN Life Science Products) in a total volume of 40 µL. Following a one hour incubation period at 55°C, samples were applied to DE81 ion exchange filter paper, washed with 0.5M sodium phosphate buffer, dried, and quantified via liquid scintillation counting. The extent of [³H]-dCTP incorporation, as measured by scintillation counting, was considered directly proportional the number of originally unmethylated sites (215).

Statistical Analysis

The mean values of each treatment group were analyzed by two-way ANOVA using SigmaStat software (SPSS) at a significance level of 5 percent. When the variance between means was significant, mean values were compared using Fisher's least significant difference procedure (254).

Results

Weight gain nor blood glucose concentrations were altered by vitamin D treatment.

Diabetic rats gained significantly less weight as compared to control animals, 65% and 59% respectively (**Table 5.1**). Further vitamin D supplementation did not affect weight gain within the diabetic and non-diabetic groups. Blood glucose concentrations were approximately 3-fold greater in STZ-treated rats regardless of vitamin D supplementation, indicating overt diabetes. Vitamin D supplementation did not significantly alter blood glucose concentrations in diabetic or non-diabetic rats compared to their respective controls.

Vitamin D supplementation restores homocysteine concentrations in diabetic rats. There was a marked decrease in plasma homocysteine concentrations in diabetic rats (**Figure 5.2**). Diabetic rats exhibited only 40% of the plasma homocysteine concentrations evident in control animals. Moreover, the hypohomocysteinemia that resulted in diabetic rats was

prevented by vitamin D supplementation. Vitamin D alone resulted in no significant change in plasma homocysteine concentrations.

Vitamin D supplementation activates GNMT in diabetic rats, but does not necessarily alter abundance. Hepatic GNMT activity was induced in diabetic rats 1.3-fold, as compared to control rats (**Figure 5.3A**). However, vitamin D supplementation in diabetic rats induced the activity of the enzyme above activity levels exhibited by rats treated with STZ alone (1.3-fold) and 1.7-fold as compared to controls. A diabetic state similarly induced GNMT protein abundance 3-fold and 4-fold in diabetic rats treated with vitamin D, as compared to the control group (**Figure 5.3B**). However the difference in mean values between the diabetic groups did not reach significance ($p = 0.1$).

SAM-dependent PEMT activity was increased in diabetic rats but vitamin D treatment was without effect. Hepatic PEMT activity was elevated 1.5-fold in diabetic rats as compared to activity levels for control animals (**Figure 5.4**). Vitamin D supplementation alone had no effect on PEMT activity. However, in vitamin D-treated diabetic rats, PEMT activity was reduced to 80% of the activity level exhibited in STZ-treated rats alone. Moreover, this value was not significantly different from the activity level in the control group, nor was it significantly different from the mean of the STZ-treated rats ($p = 0.1$).

Remethylation did not restore plasma homocysteine concentrations in vitamin D-treated diabetic rats. Differential enzyme activity induction was evident in STZ-treated rats (**Table 5.2**). Folate-independent hepatic BHMT activity increased 1.4-fold and 1.6-fold in diabetic and vitamin D-supplemented diabetic rats, respectively, as compared to the control group. Interestingly, vitamin D treatment alone effectively reduced BHMT activity 21%. Alternatively, STZ-treatment reduced the activity of the folate-dependent enzyme methionine synthase 73% and 86% in vitamin D supplemented diabetic rats as compared to the control animals. Vitamin D treatment alone or in diabetic rats did not significantly alter hepatic MS activity.

A diabetic state induced SAM-dependent global DNA hypomethylation, regardless of vitamin D treatment. Both diabetic and vitamin D-supplemented diabetic rats exhibited a reduction in global DNA methylation 1.9- and 2.0-fold, respectively, as compared to control rats (**Figure 5.5**). Vitamin D supplementation alone had no effect on methylation status.

Further, vitamin D supplementation in diabetic rats did not alter methylation status, as compared to diabetic rats.

Homocysteine transsulfuration is not altered by vitamin D supplementation in diabetic rats. A diabetic state induced catabolic transsulfuration of homocysteine (**Figure 5.6**). Abundance of the B₆-dependent enzyme CBS, which initiates transsulfuration, was induced 1.7-fold and 1.9-fold in diabetic and vitamin D-treated diabetic rats, respectively, as compared to control animals. Similarly, plasma glutathione concentrations were elevated approximately 1.6-fold in STZ-induced diabetic rats compared to control rats. However, vitamin D supplementation alone or in combination with STZ-treatment did not significantly alter homocysteine transsulfuration as compared to their respective controls.

Discussion

It has been well established that the interrelated homocysteine, folate, and methyl group metabolic pathways are critical in the prevention of a number of pathologies. Thus it is paramount to understand the various factors that regulate these metabolic processes to promote optimal health. We and others (101, 123, 198, 219, 220, 307) have shown that a chemically-induced type 1 diabetic state leads to the disruption of these interrelated pathways in hepatic tissue. Specifically STZ-induced diabetic rats have reportedly altered transmethylation as evident by increased GNMT activity, mRNA, and protein abundance, PEMT activity and abundance, and hepatic DNA hypomethylation (101, 197, 198, 303). These animals also exhibited decreased folate-dependent remethylation and increased folate-independent remethylation (198, 199, 220). Furthermore, in diabetic rats with normal renal function, homocysteine transsulfuration is increased ultimately resulting in reduced plasma homocysteine concentrations (123, 198). A number of studies indicate a diabetic state is also altered by vitamin D status. Vitamin D deficiency has reportedly predisposed individuals and animals to both type 1 and 2 diabetes (173). Impaired insulin secretion and glucose intolerance have also been reported in both humans and animals with vitamin D deficiency (37, 50, 202), but restoring vitamin D levels reverses these abnormalities (131). Vitamin D supplementation in infants and in the genetically predisposed NOD mouse, has also been indicated in the reduction of type 1 diabetes development (7, 121, 175, 259). Thus the goal

of this study was to determine if vitamin D supplementation impacts diabetes-perturbed methyl group metabolism.

The most interesting finding in our study was the hypohomocysteinemia that resulted in diabetic animals and the restoration that followed calcitriol supplementation. In this acute stage of diabetes without renal complications hypohomocysteinemia is commonly reported in both animals and humans with type 1 and 2 diabetes (123, 198, 224, 299). However as renal complications ensue in chronic diabetes, hyperhomocysteinemia results in both type 1 and 2 diabetics (118, 224), an independent risk factor for cardiovascular disease (132).

Future studies should be conducted to determine the effect of vitamin D supplementation in chronic diabetic patients and animals with nephropathy. In support of these findings, low serum concentrations of 1, 25-(OH)₂D₃ and its precursors are commonly reported in diabetics (10, 225, 243) which may result from increased urinary loss in diabetic nephropathy (204, 273, 287). Thus it is plausible that a diabetic state may be altering vitamin D metabolism; indicating diabetics may already have an increased requirement for vitamin D. Further, epidemiological studies have indicated an association between low vitamin D status and an increased risk for metabolic syndrome, peripheral arterial disease, and ultimately cardiovascular disease (87, 157, 168, 184). Thus, vitamin D may prove to be an important tool in lowering both homocysteine concentrations and the risk of cardiovascular disease in diabetics.

Reduced homocysteine concentrations in diabetic rats is commonly the result of both increased transsulfuration and remethylation of the amino acid, as evident by increased CBS, γ -cystathionase, and both activity and mRNA levels of BHMT (123, 197, 219). Our study also supported these findings. In addition, we found elevated circulating glutathione concentrations in diabetic rats, again indicating increased catabolism of homocysteine in diabetic animals. However, there were no significant changes in these enzymes in diabetic rats that received vitamin D supplementation, which does not explain the restored homocysteine concentrations. Interestingly, vitamin D supplementation alone significantly decreased BHMT activity, although there has been no reports indicating a vitamin D-response element in the promoter region for BHMT. Similar to previous findings (197),

folate-dependent remethylation decreased in diabetic animals and was not altered significantly with vitamin D treatment.

Altered transmethylation is also commonly reported in diabetic rats. We report similar findings in diabetic animals, with increased GNMT activity and abundance, global DNA hypomethylation, and increased activity of PEMT. However vitamin D supplementation increased GNMT activity further in diabetic rats, which may account for the return of homocysteine concentrations. There was also a trend for the induction of GNMT protein level in vitamin D-supplemented diabetic rats over that which was exhibited in diabetic rats. Retinoic acid (RA), which acts similarly to $1, 25(\text{OH})_2\text{D}_3$ as a transcription factor, has also been reported to increase GNMT activity and abundance (231). There are no reports however that GNMT contains a response element for either transcription factor. Further, in previous studies RA alone was capable of inducing GNMT (198, 231). Alternatively, vitamin D alone did not alter GNMT activity. RA has also been reported to increase folate-dependent remethylation through the activity of MS (208), but vitamin D treatment had no effect on MS activity. Thus the two compounds may exert their effect through distinct mechanisms.

Induction of GNMT would be expected to result in loss of methyl groups for additional transmethylation reactions. DNA methylation status did support this assumption; however vitamin D supplementation did not induce any additional hypomethylation to mimic the induction of GNMT. The DNA hypomethylation that resulted in diabetic rats was not significantly altered by vitamin D supplementation. Although, it seems vitamin D supplementation may have restored PEMT activity, a primary user of SAM-derived methyl groups (126). This trend however did not reach significance.

Since blood glucose concentrations were not significantly altered in the vitamin D-treated diabetic animals, future studies need to focus on determining the ideal treatment period and dose. It will also be ideal in future studies to examine the effect of vitamin D in diabetic patients and animals with nephropathy, possibly using a genetic model of diabetes, to further elucidate its effectiveness in restoring homocysteine concentrations. Additionally, identifying the underlying mechanism for its action will also be vital in future studies. Lack of insulin, excess counter-regulatory hormones to insulin, or elevated blood glucose may be

logical connections, since vitamin D depletion results in impairment of insulin secretion and insulin is a probable regulator of homocysteine metabolism.

In summary, we have reported that type 1 diabetes disrupts homocysteine and methyl group metabolism and that vitamin D status may influence this disruption. Since similar disruptions occur in type 2 diabetes (299) this may have future implications regarding vitamin D requirements in a diabetic state. This may be especially significant for those with polymorphisms in proteins critical in both homocysteine and vitamin D metabolism, such as the vitamin D receptor and CBS.

Footnotes

¹Nieman KM², Nieman MK³, Schalinske KL². Vitamin D supplementation maintains normal homocysteine metabolism in diabetic rats. *FASEB J* 21:226.2, 2007 (abstr.). This research was funded by the American Diabetes Association and the Martin Fund at Iowa State University.

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Tables

Table 5.1

Cumulative weight gain and blood glucose concentrations in rats treated with calcitriol (D) and/or streptozotocin (STZ)¹

	Cumulative Weight Gain (g)	Blood glucose (mM)
Control	137 ± 4^a	9.9 ± 1.3^a
D	130 ± 5^a	12.3 ± 1.3^a
STZ	90 ± 6^b	26.6 ± 1.6^b
D+STZ	81 ± 7^b	31 ± 0.1^b

¹Values are means \pm S.E. (n = 4-6). Data in each column with distinct letters are significantly different ($p \leq 0.05$).

Table 5.2

Hepatic activity of homocysteine remethylation enzymes, betaine-homocysteine S-methyltransferase (BHMT) and methionine synthase (MS), in rats treated with streptozotocin (STZ) and calcitriol (D)¹

	BHMT (units/mg protein)	MS (pmol/min•mg protein)
Control	108 ± 4 ^a	55 ± 6 ^a
D	85 ± 4 ^b	47 ± 7 ^a
STZ	153 ± 13 ^c	15 ± 2 ^b
D+STZ	174 ± 12 ^c	8 ± 2 ^b

¹Values are means ± SE (n = 4-6). Distinct letters within each column indicate a significant difference ($p \leq 0.05$).

Figure Legends

Figure 5.1

Homocysteine metabolism. Homocysteine is synthesized from methionine, which is first activated to S-adenosylmethionine (SAM). SAM, the primary methyl group donor provides methyl groups in a number of transmethylation reactions ($X \rightarrow X-CH_3$). Following methyl group donation S-adenosylhomocysteine (SAH) is synthesized. SAH is further hydrolyzed, forming homocysteine. The abundant cytosolic protein, glycine N-methyltransferase (GNMT), optimizes the SAM:SAH ratio by utilizing methyl groups from SAM forming sarcosine and SAH from glycine. Phosphatidylethanolamine N-methyltransferase (PEMT) is also a major consumer of SAM-dependent methyl groups in the synthesis of phosphatidylcholine (PC) from phosphatidylethanolamine (PE). Homocysteine can either be remethylated to methionine or irreversibly catabolized through the transsulfuration pathway. Remethylation of homocysteine occurs through both folate- and betaine-dependent pathways. Folate-dependent remethylation occurs through the activity of the B₁₂-dependent enzyme, methionine synthase (MS). A methyl group is donated from 5-methyltetrahydrofolate (5-CH₃THF) resulting in tetrahydrofolate (THF) and methionine. Homocysteine can also be remethylated with the donation of a methyl group from betaine, which is formed following the oxidation of choline. Methionine and dimethylglycine (DMG) result through the activity of betaine-homocysteine S-methyltransferase (BHMT). Alternatively homocysteine can be catabolized to cysteine which is initiated by the B₆-dependent enzyme cystathionine β -synthase (CBS). Cysteine can then be used in the synthesis of glutathione.

Figure 5.2

Vitamin D supplementation maintains homocysteine concentrations in streptozotocin (STZ)-induced diabetic rats. Rats were acclimated to their diet for 11 days. The following day, the animals began an oral treatment regimen of 500 ng/kg body weight 1,25-dihydroxyvitamin D₃ (calcitriol, D) or the vehicle corn oil, every other day for the remainder of the study. On day 21, the animals were given a single injection of streptozotocin (STZ, 60 mg/kg body weight) or the vehicle 10 mM citrate buffer (pH 4.5). Plasma samples were collected and total homocysteine concentrations were determined by HPLC and fluorometric

detection as described under “Materials and Methods.” Data are expressed as means \pm S.E. ($n = 4-6$) and bars labeled with distinct letters are significantly different ($p \leq 0.05$).

Figure 5.3

Hepatic glycine *N*-methyltransferase (GNMT) induction in vitamin D-treated diabetic rats. Liver samples were collected from the same animals as described for Figure 4.2 and GNMT activity and abundance were determined as described under the “Materials and Methods” section. **A:** Hepatic GNMT activity in both diabetic and non-diabetic rats supplemented with vitamin D or the vehicle corn oil. Data are expressed as means \pm SE ($n = 4-6$) and bars denoted with different letters are significantly different ($p \leq 0.05$). **B:** GNMT abundance was determined in the same animals as described above using Western blot analysis and a polyclonal GNMT antibody. A representative immunoblot is pictured and the data are displayed in the graph. Data are expressed as percent of the control \pm S.E. ($n = 4-6$) and bars labeled with different letters indicate a significant difference ($p \leq 0.05$).

Figure 5.4

Induction of hepatic phosphatidylethanolamine *N*-methyltransferase (PEMT) in streptozotocin (STZ)-induced diabetic rats may be prevented by vitamin D supplementation. Microsomal fractions of liver were collected to determine PEMT activity from the animals as described for Figure 4.2. PEMT activity was assessed as described under the “Material and Methods” section in both diabetic (STZ) and non-diabetic animals treated with supplemental vitamin D (calcitriol, D), or the vehicle. Data are expressed as means \pm S.E. ($n = 4-6$) and bars denoting distinct letters are significantly different ($p \leq 0.05$).

Figure 5.5

Global DNA hypomethylation in streptozotocin (STZ)-induced diabetic rats. Liver was collected for DNA isolation from both diabetic (STZ) and non-diabetic animals treated with supplemental vitamin D or the vehicle corn oil, which are the same animals as described for Figure 4.2. Global methylation status was determined using [^3H]-dCTP, where incorporation of radiolabeled dCTP is directly proportional to unmethylated sites as described in the “Materials and Methods” section. These data are expressed as means \pm S.E. ($n = 4-6$) and bars displaying distinct letters indicate a significant difference ($p \leq 0.05$).

Figure 5.6

Homocysteine transsulfuration is not altered in streptozotocin (STZ)-induced diabetic rats treated with supplemental vitamin D (calcitriol, D), as evident by hepatic cystathionine β -synthase (CBS) abundance and plasma glutathione concentrations. Liver and plasma samples were collected from the same animals as described for Figure 4.2. CBS abundance was assessed by Western blot analysis using a polyclonal CBS antibody and displayed in a representative blot. Glutathione concentrations were determined using HPLC with fluorometric detection. Both analyses are described under the “Material and Methods” section. Data are expressed as means \pm S.E. ($n = 4-6$) and displayed as a percent of the control group mean. Bars denoted with different letters are significantly different ($p \leq 0.05$).

Figures

Figure 5.1

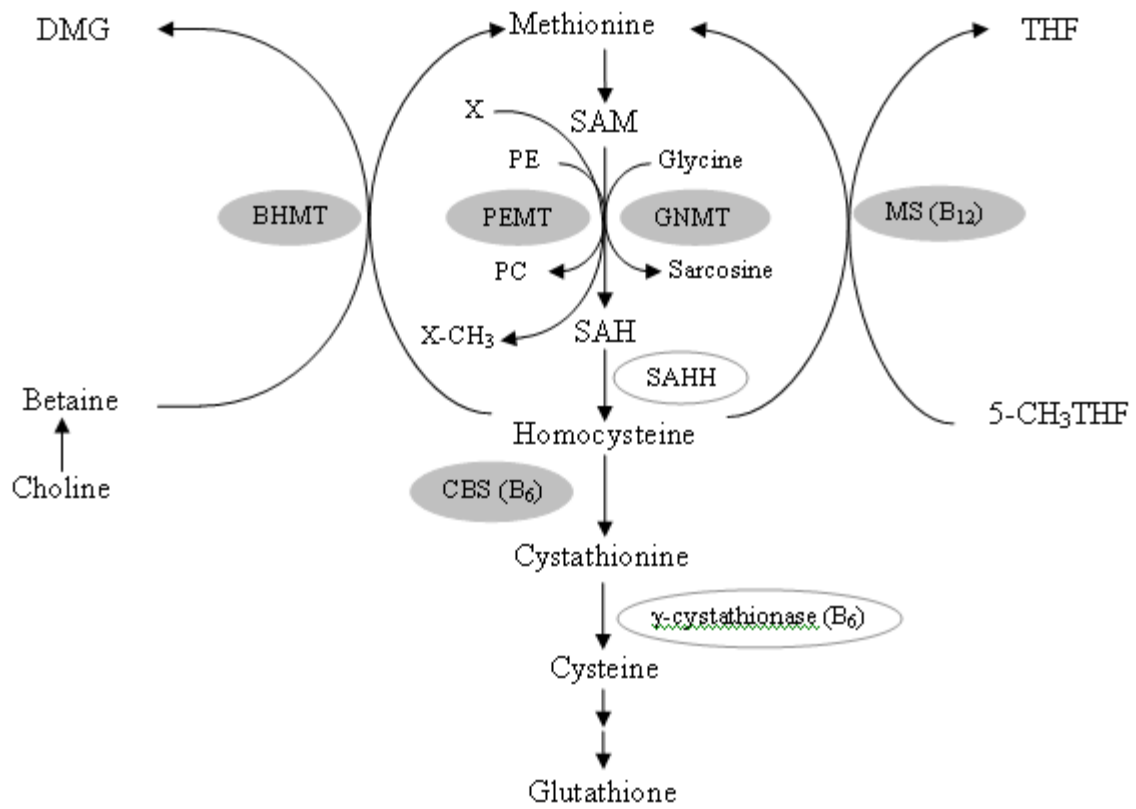


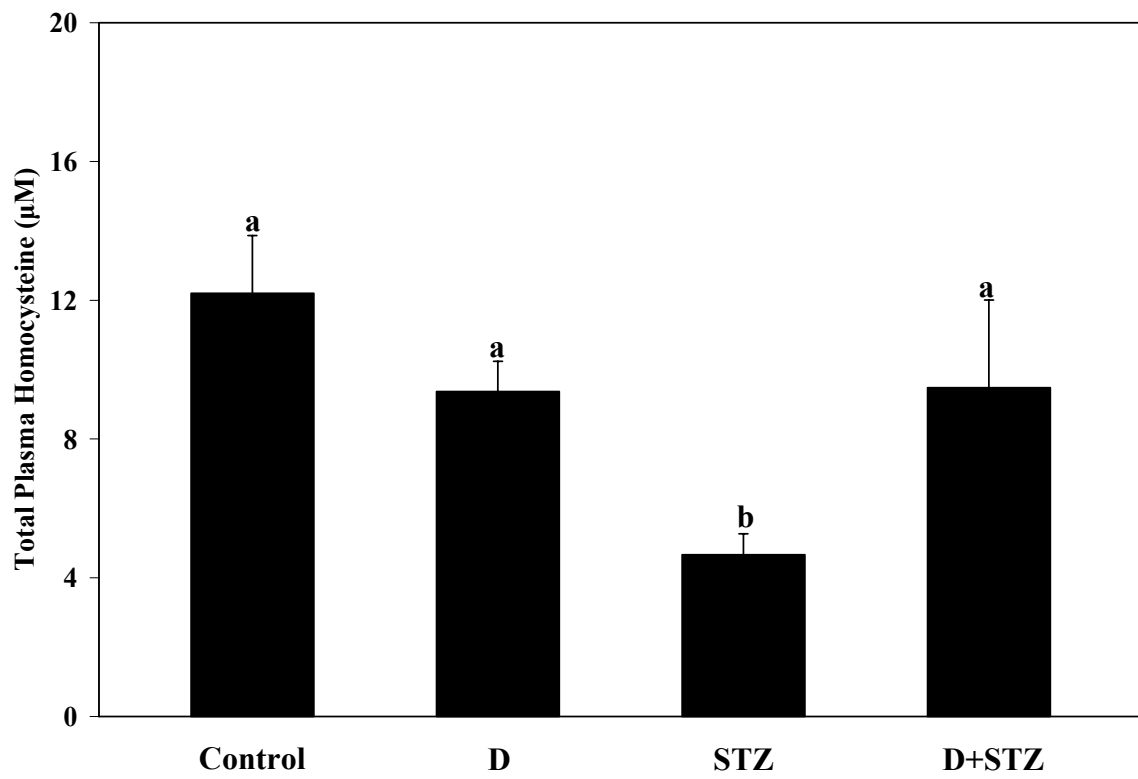
Figure 5.2

Figure 5.3A

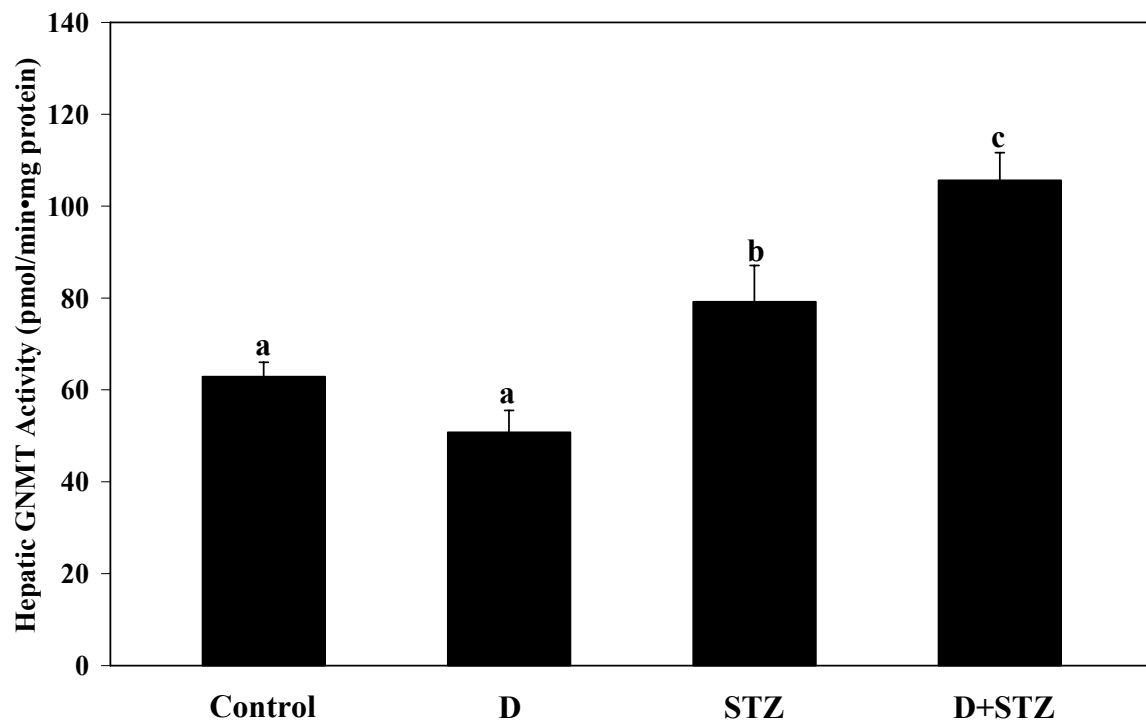


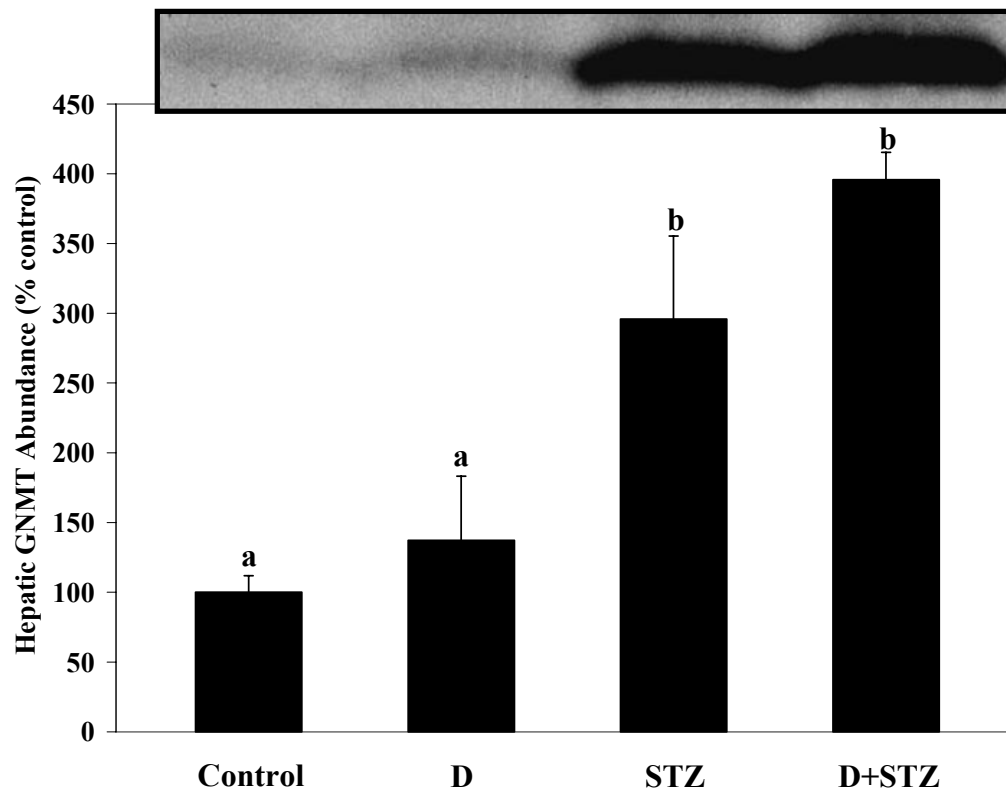
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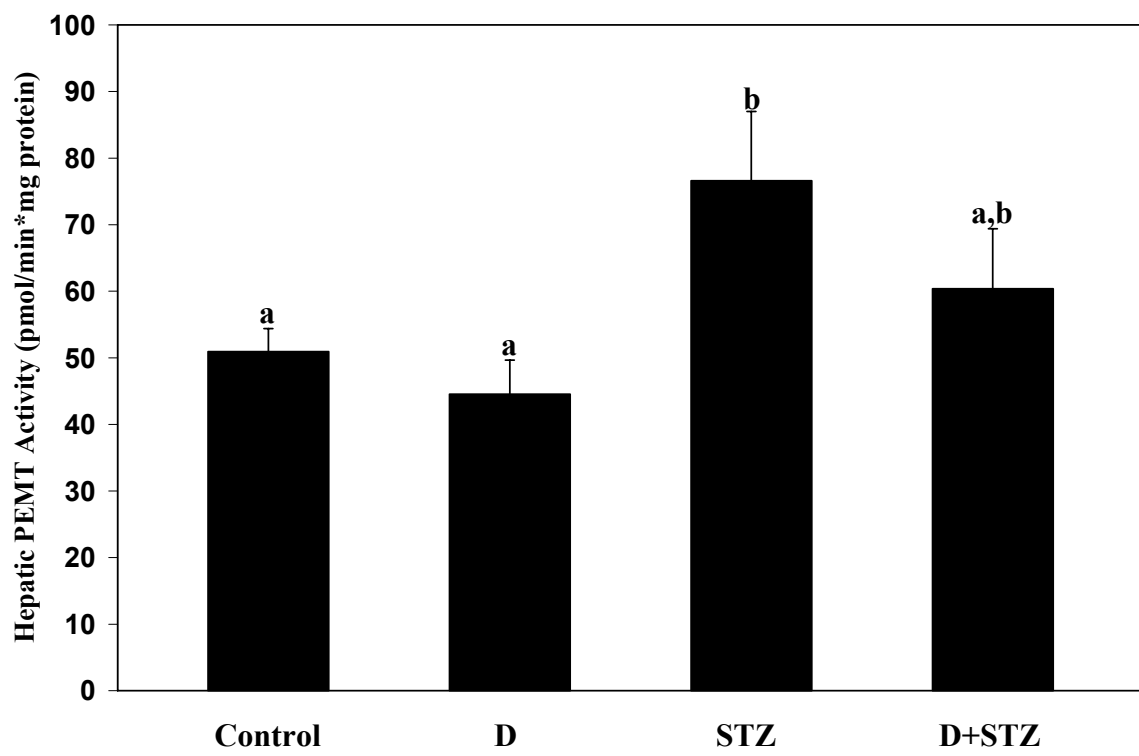
Figure 5.4

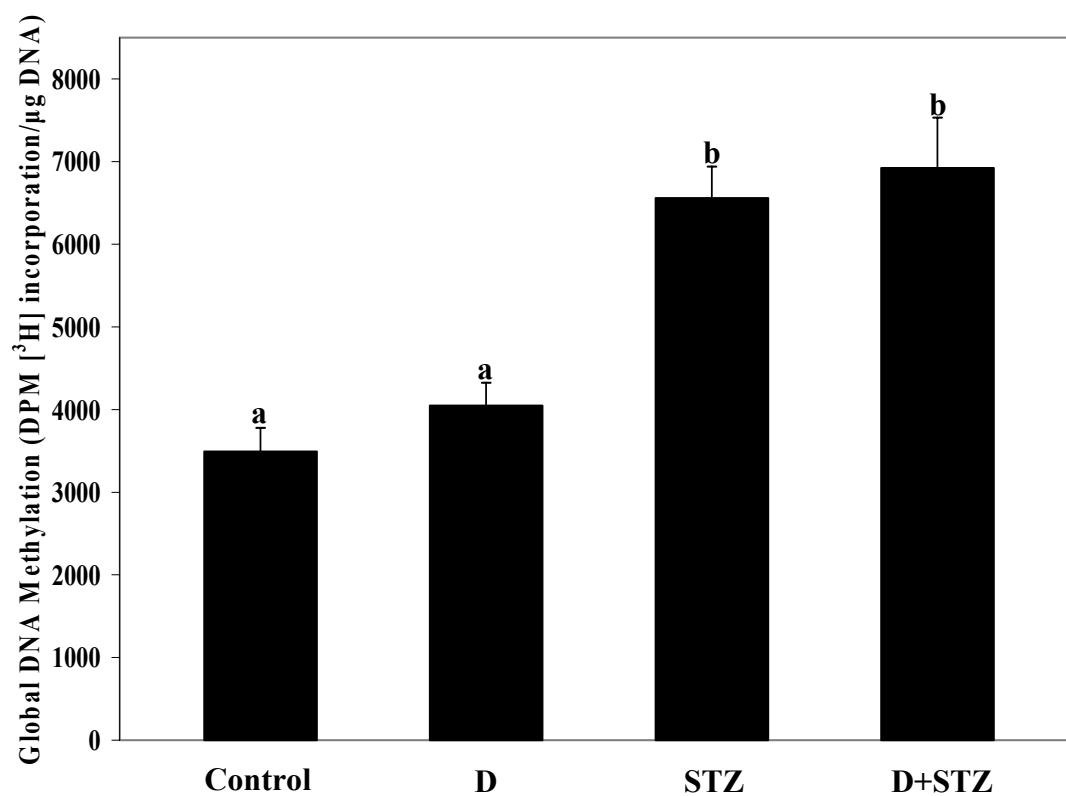
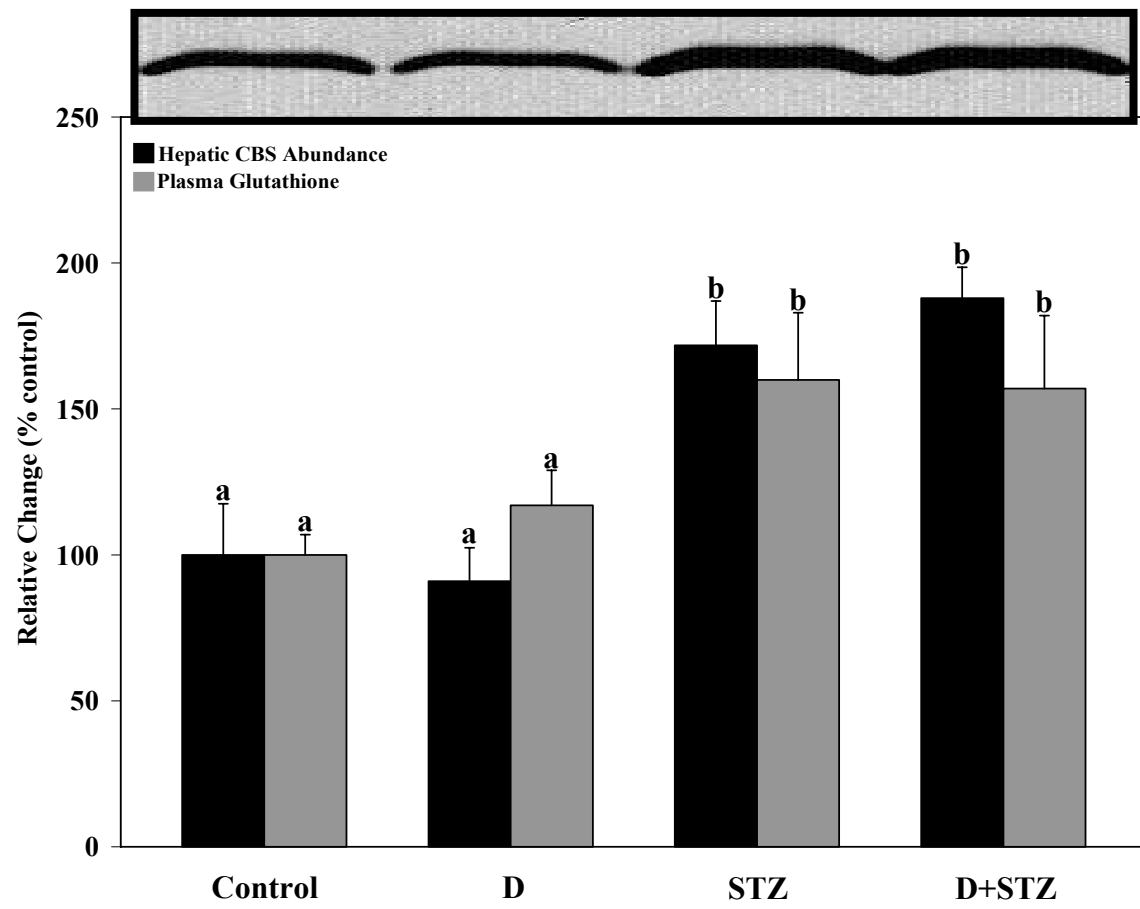
Figure 5.5

Figure 5.6



CHAPTER 6: GENERAL CONCLUSIONS

Folate, homocysteine, and methyl group metabolism function in concert to provide activated one-carbon units in the modification and synthesis of essential biological compounds. Therefore, interruptions in these vital pathways may have adverse implications in health and disease. This document further elucidates modifications in methyl group metabolism in a streptozotocin-induced diabetic state, characterized by reduced circulating insulin and elevated counter-regulatory hormones to insulin, essentially a gluconeogenic state. In addition we have investigated several different means (*i.e.* folate, vitamin D, and insulin administration) of preventing perturbation of homocysteine and methyl group metabolism in a diabetic state.

Following insulin administration, the elevation in GNMT activity and hypohomocysteinemia in diabetic rats was completely prevented by insulin treatment. Additionally, disruptions in methionine synthase (MS), PEMT, and DNA methylation were also prevented by insulin injections. Similar findings have been reported in a type 2 diabetic model, therefore it is suspected that glucose or insulin levels may represent a regulatory signal to modify GNMT and homocysteine. Moreover, this study suggests perturbations in folate, homocysteine, and methyl group metabolism result from a diabetic state as opposed to complications of streptozotocin toxicity.

The activities of GNMT, phosphatidylethanolamine *N*-methyltransferase (PEMT), and betaine-homocysteine *S*-methyltransferase (BHMT) were increased approximately 2-fold in the diabetic rat liver; folate deficiency resulted in the greatest elevation in GNMT activity. This elevation is likely due to a lack of folate which would otherwise impose posttranslational inhibition of GNMT. Although these results would suggest that adequate folate status has a positive effect under diabetic conditions, supplemental folate did not confer any added benefit at the level tested. However this does stress the importance of adequate folate, especially in diabetics. The abundance of GNMT protein and mRNA, as well as BHMT mRNA, were also elevated in diabetic rats. Thus perturbation of homocysteine and methyl group metabolism in a diabetic state is likely at the level of transcription. Hyperhomocysteinemia in folate-deficient rats was attenuated by streptozotocin, probably owing in part to increased BHMT expression. These results indicate

that a diabetic state profoundly modulates methyl group, choline, and homocysteine metabolism, and folate status may play a role in the extent of these alterations.

Vitamin D supplementation prevented hypohomocysteinemia in diabetic rats, which is likely due to an induction of GNMT. It did not prevent other diabetes-mediated alterations in homocysteine remethylation or transsulfuration. Taken together, it appears that vitamin D supplementation may be beneficial by maintaining normal homocysteine metabolism disrupted by a diabetic state.

Approximately seven percent of the American people are affected by diabetes. The results reported in this document may have significant implications for complications associated with this devastating disease. Many questions remain and need to be addressed by future research. First, it will be important to determine if a greater reduction in GNMT activity can be achieved with higher doses of folate, a different route of supplementation, and/or longer treatment times. Secondly, it will also be necessary to determine the mechanism of action of vitamin D. Insufficient vitamin D levels are often reported in diabetic humans, so it will be critical to determine the proper treatment regimen. In the work described here and in combination with other studies, it seems that a lack of insulin, increased glucose, or increased circulating counter-regulatory hormones to insulin may be a signal for regulating homocysteine and methyl group metabolism. This regulation likely occurs at the level of transcription since BHMT, CBS, and GNMT mRNA abundance have all been reportedly increased in type 1 diabetic rats. BHMT and CBS mRNA are also elevated in a type 2 diabetic rat model. However there are currently no reports in the literature of glucocorticoid-response elements in these genes. Thus it is plausible that the regulatory mechanism involves an intermediate or the regulation is at the level of mRNA stability. It will also be critical to determine the efficacy of the regulatory strategies presented, in genetic models of type 1 and type 2 diabetes, as opposed to the chemically-induced model used in these studies. Moreover, the evidence presented here indicates diabetics may have an increased requirement for nutrients such as choline, folate, other B vitamins involved in one-carbon metabolism (*e.g.* B₁₂ and B₆), and possibly vitamin D. This will be particularly important in diabetics that express polymorphisms in the enzymes involved.

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