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Retinoid- and diabetes-induced aberrations of methyl group and homocysteine metabolism including alterations of epigenetic regulation

by

Kelly T. Williams

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Nutritional Sciences

Program of Study Committee:

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2009

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LIST OF ABBREVIATIONS

5-CH_3-THF	5-methyltetrahydrofolate
5,10-CH_2-THF	5,10-methylenetetrahydrofolate
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BHMT	betaine-homocysteine *S*-methyltransferase
CBS	cystathionine β-synthase
CGL	cystathionine γ-lyase
CTα	cytidylyltransferase-α
DMG	dimethylglycine
DNMT	DNA methyltransferase
GAMT	guanidinoacetate methyltransferase
GNMT	glycine *N*-methyltransferase
HCC	hepatocellular carcinoma
MAT	methionine adenosyltransferase
MBD	methyl-binding domain (protein)
MeCP2	methyl-CpG-binding protein 2
MS	methionine synthase
MTHFR	methylenetetrahydrofolate reductase
PC	phosphatidylcholine
PE	phosphatidylethanolamine

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PEMT	phosphatidylethanolamine *N*-methyltransferase
RA	all-*trans*-retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
SAH	*S*-adenosylhomocysteine
SAHH	*S*-adenosylhomocysteine hydrolase
SAM	*S*-adenosylmethionine
STZ	streptozotocin
THF	tetrahydrofolate
ZDF	Zucker (type 2) diabetic fatty (rat)

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CHAPTER 1: Introduction

Research problem

It is our ***goal*** to further the understanding of methyl group and homocysteine metabolism as it relates to health and disease. This necessitates investigation into the factors that alter methyl group metabolism and identification of potential adverse effects of these metabolic aberrations. Previous research within our lab group and by others has demonstrated that both administration of retinoids or a diabetic condition acutely perturbs hepatic methyl group and homocysteine metabolism. Specifically, both conditions upregulate glycine *N*-methyltransferase (GNMT), an enzyme proposed to be a key regulator of methyl group supply and utilization. ***It was our <u>hypothesis</u> that sustained induction of GNMT, by either retinoids or a diabetic condition, would lead to a functional methyl deficiency, whereby methyl groups would be unavailable for other methyltransferase reactions, such as DNA methylation.*** The methylation of DNA is an important component of epigenetic regulation of the genome. Epigenetic events, such as altered DNA methylation status, cause heritable changes in gene expression without changing the DNA sequence. Epigenetic marks are also associated with the maintenance of genome stability. We expected that changes in epigenetic regulation, i.e. DNA methylation would be associated with adverse effects, such as hepatotoxicity. Aberrant epigenetic regulation also represents a possible mechanistic link to the development of the secondary complications of diabetes, which will be addressed in future studies. The ***aim*** of the studies presented here was to characterize methyl group and homocysteine metabolism by assessing key regulatory enzymes and metabolites, as well as epigenetic regulation by DNA methylation, in rats treated with all-*trans*-retinoic acid (RA) for up to six months, as well as in the streptozotocin (STZ)-induced (type 1) diabetic and Zucker (type 2) diabetic fatty (ZDF) rats.

Significance

The results of these studies offer insight into the safety of RA treatments and the pathogenesis of type 1 and type 2 diabetes. This knowledge could impact the course of treatment for many patients. Retinoids are widely used for treatment of dermatological

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conditions and in cancer chemotherapy, but these treatments have many side effects and safety of the retinoid compounds is still a matter of debate. Diabetes has been established as a serious public health concern with estimates that 12% of the U.S. population has the disease and the prevalence of diabetes is rising, both in the U.S. and worldwide. Diabetes is associated with increased morbidity and mortality due to metabolic perturbations of the disease and the development of secondary complications including cardiovascular disease, nephropathy, neuropathy, and retinopathy. By furthering the understanding of methyl group and homocysteine metabolism in these conditions, it is our hope that this information might be used for the formulation of appropriate dietary therapies to minimize adverse effects and development of secondary complications.

Dissertation organization

Following a review of the literature, this dissertation will include three chapters consisting of manuscripts that have been prepared for publication in peer-reviewed journals. The first article will be submitted to Hepatology and presents an investigation of the effects of chronic retinoid treatment on hepatic methyl group metabolism, DNA methylation status, and general markers of toxicity in rats. The second manuscript details a time course study of aberrant methyl group metabolism and DNA methylation in the STZ-induced rat model of type 1 diabetes. This article was published in the Journal of Nutrition in November 2008. The third, and final, paper describes the characterization of methyl group and homocysteine metabolism, as well as epigenetic regulation in the liver, kidney, and heart of the ZDF rat during early and advanced diabetic conditions. This article has been prepared for publication in the American Journal of Physiology – Endocrinology and Metabolism. Following this third manuscript will be an overall summary and conclusions statement which presents a thorough discussion of the results, proposes potential regulatory factors, and suggests potential future research directions.

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CHAPTER 2: Literature review

Methyl group and homocysteine metabolism

The metabolism of homocysteine and regulation of methyl balance are fundamental processes in the maintenance of health. Dietary methyl donors include methionine, folate, betaine, and choline. Utilization of methyl groups from these sources and the metabolism of homocysteine rely on transmethylation, remethylation, and transsulfuration pathways (**Figure 1**). Transmethylation begins with the activation of methionine to *S*-adenosylmethionine (SAM) via addition of an adenosyl group by methionine adenosyltransferase (MAT). SAM can then donate a methyl group to a variety of methyl acceptor molecules, resulting in a methylated product and *S*-adenosylhomocysteine (SAH). Homocysteine is produced by the hydrolysis of SAH by *S*-adenosylhomocysteine hydrolase (SAHH). Homocysteine can be recycled to methionine by folate/B_{12}-dependent or –independent pathways. The folate/B_{12}-dependent pathway utilizes a methyl group provided by 5-methyltetrahydrofolate (5-CH_3-THF) via the methylcobalamin cofactor of methionine synthase (MS). Methylenetetrahydrofolate reductase (MTHFR) synthesizes the 5-CH_3-THF substrate from 5,10-methylenetetrahydrofolate (5,10-CH_2-THF), which is a branch point in folate metabolism and is also used for thymidylate synthesis. Remethylation by folate/B_{12}-independent means is catalyzed by betaine-homocysteine *S*-methyltransferase (BHMT) and utilizes betaine as a methyl donor. In addition to dietary sources, betaine can be derived from choline by the action of choline oxidase; thereby choline is also indirectly a methyl donor for the BHMT reaction. As an alternative to remethylation, homocysteine may also be catabolized to cysteine by the transsulfuration pathway enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL), both of which are B_6-dependent enzymes. Understanding the regulation and physiological significance of each pathway, as well as the interrelationships between pathways, is an important and active area of research.

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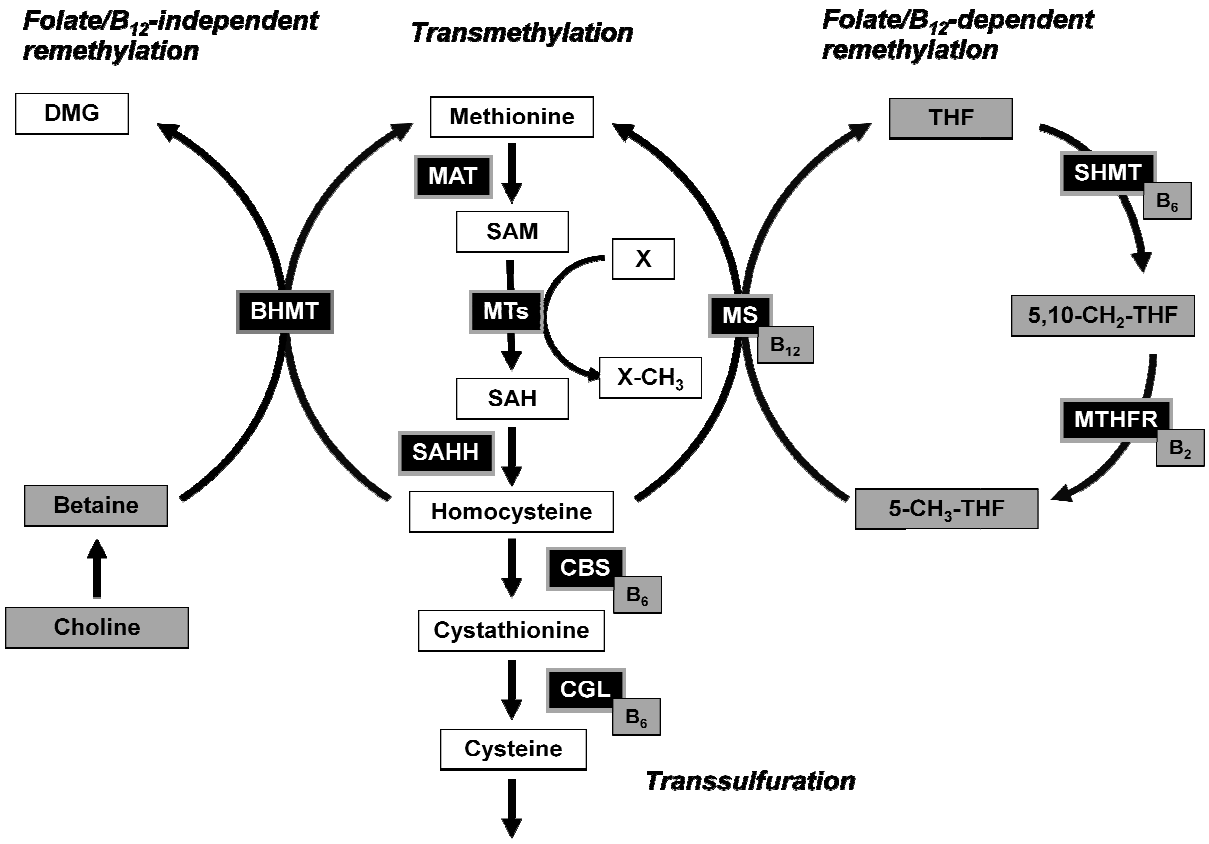

Figure 1. Methyl group and homocysteine metabolism. Enzymes shown in black boxes, B vitamin substrates and/or cofactors shown in gray boxes.

Abbreviations: 5-CH_3-THF, 5-methyltetrahydrofolate; 5,10-CH_2-THF, 5,10-methylenetetrahydrofolate; BHMT, betaine-homocystiene *S*-methyltransferase; CBS, cystationine β-synthase; CGL, cystathionine γ-lyase; DMG, dimethylglycine; MAT, methionine adenosyltransferase; MTHFR, methylenetetrathydrofolate reducttase; MTs, methyltrasnferases; SAH, *S*-adenosylhomocysteine; SAHH, *S*-adenosylhomocysteine hydrolase; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate; SHMT, serine hydroxymethyltransferase; X, methyl acceptor

Transmethylation

The condensation of methionine with adenosine is catalyzed by any of the three isoforms of MAT. The isoforms of MAT are functionally different based on their kinetic properties and tissue-specificity (1-3). MAT I and MAT II have a low Michaelis constant (K_m), whereas MAT III has a high K_m. MAT I and MAT II function at near maximal capacity under normal intracellular methionine concentrations. In addition, unlike MAT I and II, the MAT III isoform is not subject to product inhibition by SAM and thus, is uniquely able to

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continue to use more substrate as methionine load increases. MAT II is specific to extrahepatic tissues, whereas MAT I and III are expressed in the liver. The presence of MAT III in the liver is just one of many pieces of evidence that suggests a key role for the liver in regulating methyl supply and utilization.

Following the activation of methionine to SAM by MAT, SAM can be used in a variety of transmethylation reactions. There are purported to be >100 SAM-dependent methyltransferases, over 30 of which have been characterized (4). Transmethylation reactions take place in all cells and involve the transfer of a methyl group from SAM to a methyl acceptor, resulting in the generation of the methylated product and SAH. Methyl acceptors include lipids, proteins, nucleic acids, and other small molecules. Lipids produced by transmethylation reactions include phosphatidylcholine, which is a vital component of membranes, bile acids, and lipoproteins, and involved in cell signaling (5). Methylated proteins include cellular receptors, histones, and transcription factors (6). SAM-dependent transmethylation reactions are also responsible for the methylation of non-coding and coding RNAs, as well as the establishment and maintenance of DNA methylation patterns (7,8). Other small molecules that are methylated include the neurotransmitters of the serotonin and catecholamine pathways (9) and ubiquinone in the mitochondria (10). This list highlights just a few of the biologically important molecules derived from or modified by SAM-dependent transmethylation reactions.

In addition to these varied methylated products, all transmethylation reactions produce SAH. This product can be hydrolyzed to homocysteine and adenosine by SAHH. This reaction is bidirectional and favors the formation of SAH, but proceed towards homocysteine with the removal of the products (11). Elevated levels of both SAH and homocysteine have been associated with the development of disease, including neurological disorders, vascular diseases, and renal dysfunction (12-16). Therefore, regulation of the production of these metabolites is critical.

There are three methyltransferases that have been proposed to be major contributors to the regulation of methyl group and homocysteine metabolism: guanidinoacetate methyltransferase (GAMT), phosphatidylethanolamine *N*-methyltransferase (PEMT), and glycine *N*-methyltransferase (GNMT). It has long been known that GAMT and PEMT catalyze the most quantitatively significant SAM-dependent

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transmethylation reactions under normal physiological conditions (17). GAMT facilitates the transfer of one methyl group from SAM to guanidinoacetate to form creatine, a ready source of energy for cells. Traditionally, GAMT was thought to be the largest single consumer of methyl groups from SAM (17), but Stead et al. (18) have recently summarized evidence which suggests that PEMT may be a larger consumer of methyl groups and more important contributor to elevations of homocysteine than GAMT. In addition to methodological questions regarding earlier studies, it is noted that creatine can also be supplied in the diet. Their study of creatine supplementation in rats demonstrated decreased guanidinoacetate synthesis, a 90% decrease in GAMT activity, and 25% decrease in plasma homocysteine levels (19). Subsequent studies in rats have also shown decreases in plasma homocysteine (20; Nonnecke & Schalinske, unpublished observations). However, studies in humans have provided conflicting results. Unexpectedly, two recent studies have shown creatine supplementation to be associated with increases in plasma homocysteine levels in two very different subject populations: healthy young men (21) and patients with coronary artery disease (22). Of the remainder of human studies of creatine supplementation, one found a small, but significant decrease in plasma homocysteine (23), whereas others found no effect (24) or were inconclusive due to confounding factors such as renal disease and use of dietary supplements (25).

The reaction catalyzed by PEMT – the other proposed regulator of homocysteine levels - consists of the sequential addition of three methyl groups to phosphatidylethanolamine (PE), thereby consuming three molecules of SAM and generating three molecules of SAH, as well as the product phosphatidylcholine (PC). Under normal physiological conditions, PEMT is estimated to generate one-third of PC produced and the remainder is produced via the CDP-choline pathway (26). Cytidylyltransferase-α (CTα) and PEMT knockout models have been used to determine the physiological significance of altered PEMT activity (27-30). The rate-limiting step of the CDP-choline pathway of PC synthesis is catalyzed by CTα. Therefore, knocking out CTα would be expected to increase PEMT activity for adequate PC synthesis. Accordingly, in CTα-deficient mice, PEMT activity increased 100% and methylation of PE, as measured using radiolabeled methionine, also increased 100% (27). Furthermore, plasma homocysteine levels were elevated 20-40% in CTα-deficient mice compared to controls, despite compensatory increases in BHMT activity. Likewise, transfection of PEMT into rat hepatoma cells resulted in ~1.5-fold increase in homocysteine secretion (28). Conversely, primary hepatocytes from PEMT $^{-/-}$ mice secreted

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~50% less homocysteine than cells from wild-type mice and plasma homocysteine levels were ~50% lower in both male and female mice deficient in PEMT. Deficiency of PEMT also decreased hepatic levels of choline, choline-containing phospholipids, triglycerides, and lipoproteins, as well as altered lipoprotein production and metabolism (29,30).

Whereas GAMT and PEMT have been most closely linked to homocysteine levels, GNMT is a proposed regulator of methyl group supply and utilization (31). GNMT is expressed in kidney, pancreas, and small intestine and is highly abundant in the liver, comprising up 1-3% of total hepatic protein (32). The GNMT tetramer catalyzes the addition of a methyl group to glycine, thereby forming sarcosine. Sarcosine has no clear physiological function and can be metabolized by sarcosine dehydrogenase, making it an ideal product for disposal of excess methyl groups. GNMT is also notable in that it is not subject to feedback inhibition by SAH unlike most, if not all, other known SAM-dependent methyltransferases (31). Based on these characteristics, accumulating evidence from GNMT knockout models, and a unique regulatory relationship with folate metabolism, GNMT is proposed to be a primary regulator of the SAM:SAH ratio which governs transmethylation potential.

Two GNMT knockout mouse models have recently been developed, and although there is some debate regarding completeness of the knockout and differences in the course of pathogenesis in the two models (33), animals from both models exhibit dramatic increases in the SAM:SAH ratio and development of hepatocellular carcinoma (HCC; 34-37). The Wagner group produced the first published report of a GNMT knockout mouse which was characterized by elevated hepatic methionine and SAM concentrations concurrent with decreased SAH concentrations, resulting in a 100-fold increase in the hepatic SAM:SAH ratio (34). In the GNMT knockout model developed by Chen's group, the SAM:SAH ratio was elevated 42- and 82-fold in male and female GNMT $^{-/-}$ mice, respectively (35). There was no elevation of the SAM:SAH ratio in heterozygotes in either model, despite a 50% reduction in GNMT activity in heterozygotes as reported by Luka et al (34). The development of HCC in GNMT knockout mice is postulated to be due to dysregulation of DNMTs, DNA and histone methylation, as well as perturbation of canonical signaling pathways involved in carcinogenesis including the Wnt, JAK/STAT, and MAPK pathways (36,37). Human HCC and prostate cancer have also been characterized by downregulation or lack of GNMT (38-40).

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Upregulation of GNMT has been postulated to be protective against toxins and carcinogenesis. In addition to its regulatory role in methyl group metabolism, GNMT is also a purported 4S polycyclic hydrocarbon-binding protein (41). Benzo[α]pyrene and aflatoxin or their metabolites are aryl hydrocarbons and causal agents of HCC. Upregulation of GNMT in transfected cell lines or a transgenic mouse model decreased toxin-associated DNA adduct formation and reduced cytotoxicity or prevented the development of HCC (42,43). In response to the toxins, GNMT was translocated to the nucleus and molecular modeling suggested interactions at the SAM-binding site of the dimeric form of GNMT. Earlier reports have also shown translocation of GNMT to the nucleus and measured significant GNMT activity in nucleic extracts (32), though the role of GNMT in the nucleus remains somewhat ambiguous.

Whereas GAMT does not appear to catalyze the rate-liming reaction of creatine synthesis and is proposed to respond largely to guanidinoacetate concentrations (44,45), PEMT and GNMT are subject to regulation by a wide variety of hormonal factors. Expression and/or activity of PEMT and GNMT are gender-specific or responsive to sex hormones (46,47). GNMT and PEMT are induced by a diabetic condition or treatment with glucocorticoids (32,48-52), whereas insulin treatment attenuates this effect (48,50). As another potential function for GNMT, this diabetes-induced upregulation has been proposed as a mechanism for the generation of pyruvate from methionine for gluconeogenesis, especially as GNMT expression is limited to gluconeogenic tissues (32). However, upregulation of GNMT is not specific to a diabetic state. GNMT activity and expression are also altered by growth hormone: activity and mRNA abundance are increased in the Ames dwarf mouse which lacks growth hormone, prolactin, and thyroid-stimulating hormone; this increase was attenuated by treatment with growth hormone (53,54). Likewise, triiodothyronine normalized elevations in GNMT activity by retinoids, although GNMT abundance was unaffected, suggesting posttranslational regulation; neither hypo- nor hyperthyroidism had an independent effect on GNMT activity or protein abundance (55).

There is also a unique regulatory relationship between GNMT and folate metabolism. GNMT can be bound by 5-CH_3-THF, which inhibits GNMT activity (56), specifically by inhibition of phosphorylation which enhances GNMT activity (57). However, with GNMT activity decreased, this would be expected to increase intracellular SAM levels and MTHFR is allosterically inhibited by SAM (58). When methyl group supply is high, SAM levels are

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elevated and MTHFR is inhibited, thereby decreasing the available methyl groups incoming via folate/B_{12}-dependent remethylation. Furthermore, GNMT activity is freed from inhibition by 5-CH_3-THF, resulting in lowering of SAM levels and restoration of the SAM:SAH ratio. When methyl group supply is low, SAM levels are decreased, relieving the inhibition of MTHFR and increasing 5-CH_3-THF concentrations, which inhibits GNMT activity and provides more substrate for remethylation via MS, thereby increasing the methyl pool. This reciprocal regulation of GNMT and MTHFR is thought to play a key role in transmethylation potential homeostasis.

Remethylation

Homocysteine can be remethylated by folate/B12-dependent or –independent pathways. In addition to the remethylation of homocysteine to methionine, folate metabolism is also involved in the transfer of one-carbon units for the synthesis of purines and thymidylate (59). The C2 and C8 carbons of purines are donated from the formyl group of 10-formyltetrahydrofolate in reactions catalyzed by 5-amino-4-imidazole carboxamide transformylase and glycinamide ribotide transformylase, respectively. For thymidylate synthesis, formaldehyde is transferred from 5,10-CH_2-THF to deoxyuridylate by thymidylate synthetase. Alternatively, MTHFR converts 5,10-CH_2-THF to 5-CH_3-THF, which is the methyl donor for the remethylation of homocysteine to methionine by the folate/B_{12}-dependent pathway. The folate/B_{12}-dependent pathway of remethylation appears to be active in all tissues and is essential for survival (60,61). In the attempt at developing a MS knockout in two strains of mice, Swanson et al. (61) reported that MS activity was decreased 40% in heterozygotes relative to controls, but there were minimal effects on plasma methionine or homocysteine levels. There were no viable homozygotes, with loss of the embryo occurring shortly after implantation. It is likely that the failure to survive was due, at least in part, to the effects of the “methyl trap” hypothesis, in which folate accumulates as 5-CH_3-THF due to the irreversibility of the MTHFR reaction and lack of other 5-CH_3-THF-metabolizing enzymes and thus, is unavailable for the other critical reactions requiring folate coenzymes (59). This “methyl trapping” phenomenon was first proposed after aberrations of folate metabolism were noted in patients and rats lacking adequate vitamin B_{12}, an essential cofactor for MS activity. Unlike the MS knockout model, both hetero- and homozygous

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MTHFR knockout mice were viable and exhibited elevated plasma homocysteine levels and decreased SAM:SAH ratios in liver, brain, ovaries, and testes (62). The phenotypic effects were significantly greater in MTHFR$^{-/-}$ mice, which were developmentally delayed and had neural and vascular abnormalities in addition to the 10-fold increase in plasma homocysteine. Taking into consideration the effects on metabolites observed in the MS and MTHFR knockout models and the unique relationship between the MTHFR and GNMT, it appears that MTHFR has a more significant regulatory role than MS.

Homocysteine may also be remethylated to homocysteine by the action of BHMT in the folate/B12-independent remethylation pathway. Choline can be converted to betaine by choline oxidase and betaine aldehyde dehydrogenase in the mitochondria. Thereby, both dietary choline and betaine may serve as methyl donors for this reaction. BHMT is suggested to contribute significantly to the regulation of homocysteine levels. BHMT is primarily a liver-specific enzyme, but can also be found in the kidney in primates and pigs, with small amounts expressed in the rat kidney (61). Although there are no genetic knockout models for BHMT, *S*-(δ-carboxybutyl)-DL-homocysteine is a potent inhibitor of BHMT activity and has been used experimentally to determine the effect of BHMT on homocysteine levels (64). A single injection of *S*-(δ-carboxybutyl)-DL-homocysteine (1 mg) in mice was sufficient to decrease BHMT activity 90% and increase homocysteine levels by greater than 100% for 1-8 hrs. There was no effect on MS and CBS activities, suggesting that changes in homocysteine could be attributed specifically to the decrease in BHMT activity.

MS and BHMT are regulated by many of the same factors that influence the SAM-dependent methyltransferases. The effects of a diabetic condition on MS, MTHFR, and BHMT are varied and will be discussed in depth in subsequent sections. In the growth hormone-deficient Ames dwarf mouse, the mRNA expression of BHMT was upregulated, whereas MS expression was suppressed (54). MTHFR activity in the liver and/or kidney is affected by sex steroids, thyroid, and growth hormone (60). BHMT activity has also been shown to be suppressed by triiodothyronine (55) and is likely affected by testosterone and estrogens, but much remains to be learned about the hormonal regulation of BHMT (65).

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Transsulfuration

Transsulfuration is the irreversible catabolic pathway for homocysteine to cysteine and is specific to the liver, kidney, pancreas, intestine, and brain (1). As the only reaction resulting in the catabolism of homocysteine, CBS has also been implicated in the regulation of plasma homocysteine concentrations. The effect of CBS upregulation on serum homocysteine levels was investigated by Wang et al. (66) using a transgenic mouse model. By giving mice supplemental zinc, transcription of the CBS transgene under the regulation of the metallothionein promoter was stimulated, which resulted in increased CBS activity and expression in the liver and kidney with concurrently decreased serum homocysteine concentrations. The homocysteine-lowering effect of CBS upregulation was also observed when mice were fed a high methionine-low folate diet, which successfully induced hyperhomocysteinemia in wild-type mice. Conversely, mice that are completely deficient in CBS had plasma homocysteine concentrations 40-fold greater than their wild-type counterparts, whereas CBS activity decreased 50% and plasma homocysteine concentrations increased 2-fold in the heterozygotes (67).

CBS and CGL are also subject to regulation, by diabetes as well as other hormones and cellular conditions. CBS and CGL activities were elevated in the Ames dwarf mouse liver (53). The effect of increased activities of these enzymes in the Ames dwarf mouse were studied further, which revealed increased flux through the transsulfuration pathway in the liver, kidney and brain, as well as lower plasma homocysteine concentrations (54). CBS activity and transsulfuration flux are also enhanced in response to increased SAM concentrations (68) and under oxidative conditions (12). This is likely due to the need for disposal of excess methionine and/or generation of cysteine, which can be used for the production of a variety of important intracellular metabolites, including the antioxidants glutathione and taurine. Glutathione and taurine are involved in redox homeostasis and are considered a key defense against intracellular oxidation. Cysteine is also a precursor to hydrogen sulfide, which is a transmitter with neurological, intestinal, and cardiovascular effects (69).

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Methyl balance, homocysteine, and the SAM:SAH ratio

Dietary methyl donors include methionine, folate, betaine, and choline. Methionine is an essential, sulfur-containing amino acid found in dietary proteins. Folate and betaine are utilized by the remethylation pathways for donation of methyl groups to homocysteine for regeneration of methionine. Choline does not serve as a methyl donor directly, but it can be converted to betaine by the enzyme choline oxidase, also commonly referred to as choline dehydrogenase. Varying dietary composition with respect to dietary methyl donors can perturb folate one-carbon, methyl group, and homocysteine metabolism.

Methionine is metabolized to SAM, the primary methyl donor for transmethylation reactions and a positive regulator of transmethylation and transsulfuration reactions (1). Feeding rats high-methionine diets increased activities of MAT, GNMT, and SAHH (70,71). Transsulfuration enzymes were also upregulated, whereas MS was decreased (72). BHMT was upregulated in response to low methionine diets and this effect was enhanced by betaine or choline supplementation (72,73).

Diets deficient in one methyl donor illustrate the balance required between the pathways. Low folate may disrupt the balance that normally results from the reciprocal regulation of GNMT and MTHFR. In rats fed a folate-deficient diet, GNMT activity increased and the intracellular SAM:SAH ratio decreased in the liver and pancreas within just 2 weeks (74,75). Folate or choline deficient diets are associated with hyperhomocysteinemia and decreased concentrations of the provided methyl donor (i.e. choline deficiency results in lower hepatic folate concentrations), likely due to compensatory upregulation of the remethylation of homocysteine by the pathway utilizing the available methyl donor (76). In humans, an acute dose of betaine (3 or 6 g) was sufficient to decrease plasma homocysteine concentration up to 10% in healthy middle-aged adults (77).

Normal plasma homocysteine levels in humans range from 5-15 μmol/L (78). Mild to moderate hyperhomocysteinemia is classified as plasma homocysteine levels between 15 and 30 μmol/L, intermediate and severe hyperhomocysteinemia refers to plasma concentrations above 30 μmol/L and 100 μmol/L respectively. Disruption of homocysteine metabolism, specifically hyperhomocysteinemia, has been associated with increased risk of cancer, cerebro- and cardiovascular disease, neurological disease, osteoporosis, pregnancy complications, and birth defects (78-82). In the Hordaland Homocysteine Study of over

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18,000 patients in Western Norway, lifestyle and dietary factors such as smoking, increased coffee consumption, and low folate intake were associated with hyperhomocysteinemia, whereas there was an inverse relationship between physical activity and plasma homocysteine concentrations (82). Age, gender, renal function, and the MTHFR 677C>T polymorphism were also found to be key determinants of homocysteine levels. Plasma homocysteine concentrations were increased in men and patients that were older, had elevated creatinine levels (an indicator of renal dysfunction), and/or the T/T genotype for MTHFR 677. The MTHFR677C>T point mutation is particularly common, with prevalence of up to 20% in some populations, but polymorphic forms of GNMT, MS, SHMT, and CBS have also been identified (83,84). This raises the possibility for nutrient-gene or gene-gene interaction effects and indeed, the response of homocysteine and folate one-carbon metabolism to folate has been shown to be affected by polymorphisms (83-85) and relationships with other methyl donors are likely to exist. Inborn defects of methyl group and homocysteine metabolism have also been documented for all enzymes with the exception of BHMT and are associated with the accumulation of intermediate metabolites of their respective pathways (86).

Dietary supplementation of B vitamins with the goal of lowering plasma homocysteine levels has been studied extensively and has been shown to be effective, but whether the lowering of homocysteine levels had protective effect against morbidity and mortality is still controversial (Williams and Schalinske, BioFactors, in press). Early studies showed improved B-vitamin status lowered plasma homocysteine levels, and decreased incidence of adverse events or improvements in indicators of vascular endothelial dysfunction in patients treated with folic acid, B_{12}, and/or B_6 vs. those treated with a placebo (87-91). However, these results have largely been refuted by the predominantly negative results of subsequent trials, including the NORVIT, WENBIT, HOPE-2, VISP, VITATOPS, and VITRO studies (92-102). These studies generally had a mean follow-up time of several years and assessed a wide variety of vascular indicators and endpoints including carotid intima-media thickness and flow-mediated dilation (96), markers of arterial inflammation (93-102), need for revascularization procedures (98), occurrence of thromboembolism (97,99), occurrence of stroke and myocardial infarction (92,94,98,100,101,103), as well as overall or coronary/vascular-related mortality (92,94,95,98,100,103).

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It is not clear as to what factors may account for the disparity in these findings. The duration of treatment, B-vitamin status, and polymorphisms of enzymes involved in homocysteine metabolism could potentially play a role. Notably, the treatment period was generally longer in duration for the later studies in comparison to those conducted earlier, i.e. several years vs. weeks or months. The apparent affect of the duration of treatment is supported by the meta-analysis by Potter et al (96) in which they found that in patients post-stroke, B-vitamin treatment had positive effects in the short term, but these effects were not sustained long term. Research in this area is ongoing and several recent short term studies have demonstrated benefits of folate supplementation alone on vascular outcomes in high-risk patients (105-107). Interestingly, the data suggests that improvements are independent of the homocysteine-lowering effect of treatment, therefore other mechanisms of action should also be considered for short-term treatment effects. More data is also expected from additional long-term studies of high-risk populations which have been initiated, but are not yet complete (108,109). Although poor B-vitamin status and polymorphisms of MTHFR have been associated with elevations in plasma homocysteine levels and may impact the homocysteine-lowering response to treatment, there is little or no evidence to support that these factors may account for differential results between studies.

Vascular diseases are not the only conditions in which there have been trials of B-vitamin interventions. There appears to be no effect of homocysteine-lowering B-vitamin therapy on Alzheimer's disease and cognitive decline (110-112), and the results are conflicting regarding potential effects on bone mineral density and turnover, and fracture occurrence (113-115). However, not all findings have been negative; the Women's Antioxidant and Folic Acid Cardiovascular Study found that long-term daily treatment with folic acid, pyridoxine, and cobalamin in a high-risk population reduced the risk of age-related macular degeneration (116). Furthermore, studies in healthy individuals suggest that treatment with B vitamins may be an effective means of reducing the risk of stroke (117) and slowing the progression of early-stage atherosclerosis (118). Though the data is limited, the most important research area for the future use of treatments with the homocysteine-lowering vitamins will be identifying specific populations that are most expected to benefit from therapy, with particular emphasis on primary prevention.

Although homocysteine concentrations have been closely linked to the development of disease, some studies have suggested that SAH may be an even better biomarker (13-

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16). Maintenance of the SAM:SAH ratio is critical because this ratio serves as an indicator of transmethylation potential. Thus, important transmethylation reactions, such as the methylation of DNA and PE, are compromised when the SAM:SAH ratio is lowered (119-122). Hypomethylation of DNA was associated elevated SAH levels in particular. Conversely, elevated SAM levels in the brain of the PEMT knockout mouse were associated with hypermethylation of DNA and proteins (123). As another contradiction between the two GNMT knockout models, the Chen group reported global DNA hypomethylation using the Methylamp Global DNA Methylation Kit from Epigentek (37), whereas the group of Wagner, Luka, and Mato found genomic DNA hypermethylation – as would be expected due to the greatly increased availability of SAM – as measured by high performance capillary electrophoresis (36). In addition to the potential role of GNMT at the tissue level, interorgan metabolism of SAH may also help regulate whole body levels of SAH, thereby affecting the SAM:SAH balance. Based on the arterio-venous differences across tissues, it has recently been shown that SAH is exported from the liver, whereas 40% of circulating SAH was removed by the kidney (124). With physiological levels of plasma homocysteine and normal renal function, the kidney also removes a substantial amount of circulating homocysteine (20-50%) from the plasma, where the majority is metabolized rather than being excreted (125-127). Furthermore, homocysteine uptake and metabolism in the healthy rat kidney increases in response to experimentally-induced hyperhomocysteinemia. Clinically, there is a strong association between elevated homocysteine levels and increasing severity of renal disease (128) and transmethylation flux has been shown to be suppressed in diabetic patients with nephropathy (129). Taken together, this suggests that adequate kidney function appears to be a critical component for homeostasis of the SAM:SAH ratio and homocysteine concentrations. This also highlights the complexity of methyl group and homocysteine metabolism, and although each of the constituent pathways has been well-studied, additional efforts need to be made towards the development of a more unified theory of regulation of methyl group and homocysteine metabolism. This might be accomplished through a combination of techniques including mathematical modeling (130) and *in vivo* tracer kinetic studies (131).

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Epigenetic regulation of gene expression

The term “epigenetics” was first introduced by Waddington in the early 1940’s (132). Waddington used the term to describe the interactions between the environment and genes that governed the development of cells. Epigenetics remains an active field of investigation in developmental biology, but the concept has further evolved and the modern definition of epigenetics refers to heritable - mitotic and/or meiotic - changes in gene function that cannot be attributed to alterations of the sequence of bases (133). Epigenetic mechanisms include DNA methylation, histone modifications, and chromatin remodeling. These processes can be thought of as a level of regulation superimposed on the genome. Although epigenetic marks are heritable, they have also been shown to be vulnerable to change by environmental influences. Changes in epigenetic regulation are hallmarks of development, aging, and the pathology of many diseases.

DNA methylation is likely the most thoroughly studied epigenetic mechanism. Methylation of DNA serves many functions including contributions to genome stability, repression of parasitic elements, imprinting, X chromosome inactivation, and regulation of gene expression (8). DNA is methylated at the 5'-position of cytosine bases within the context of CpG dinucleotides, which consist of a cytosine base linked to a guanine base by a phosphodiester bond. Eighty percent of CpG dinucleotides are generally highly methylated and located in repetitive sequences and satellite DNA, whereas the other CpGs are found in dense clusters called CpG islands (8,134). Methylated regions are typically associated with tightly condensed heterochromatin. Alternatively, unmethylated CpGs are generally found in the more loosely packed euchromatin. CpG islands are associated with over 50% of known genes and are defined by an overall GC content of >60% over the range of at least 200 bases, and are found upstream of genes in the promoter, untranslated region, or exon 1 (135). Although under normal conditions most CpG islands are unmethylated, patterns of CpG methylation appear to be developmental stage- and tissue-specific (134). Aberrant global and CpG island DNA methylation has also been noted in the pathogenesis of diseases such cancer and cardiovascular disease (8,136).

DNA methyltransferases are a family of enzymes involved in the establishment and maintenance of DNA methylation patterns via SAM-dependent transmethylation. The *de novo* DNA methyltransferases that establish DNA methylation patterns are DNMT3a and 3b,

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whereas DNMT 1 serves as the maintenance methyltransferase (137). Reflecting their function, DNMT3a and 3b are most abundantly expressed in embryonic stem cells, but expression decreases with differentiation (138). There is also a third member of the DNMT3 family, DNMT3L, which is a regulatory factor that interacts with DNMT3a and 3b to enhance *de novo* methylation (103). DNMT1 functions to maintain DNA methylation patterns during cellular replication. As such, DNMT1 contains a replication foci targeting sequence, interacts with the transcriptional machinery, and the methyl-DNA-binding site favors hemimethylated DNA (137,140).

Most DNA does not exist freely in the nucleus. Rather, it is wrapped around an octomer of histones, forming nucleosomes, and then is further condensed into the higher order structure of chromatin. There are extensive and complex interactions between DNA methylation, histone modifications, and chromatin remodeling that affects the structure of DNA, which in turn is related to genomic stability and regulation of gene expression (141-143). The proteins that help bridge the gap between DNA methylation and the formation of heterochromatin are the family of methyl-CpG-binding domain (MBD) proteins (141). The MBD family consists of MBD1-4 and methyl-CpG-binding protein 2 (MeCP2). In addition to the common MBD, all MBD proteins have been shown to be capable of mediating transcriptional repression, primarily through the recruitment of other regulatory proteins that reinforce the DNA methylation signal with histone modifications and chromatin remodeling. MBD1 interacts with the SET and Suv histone methyltransferases. MBD2 and MBD3 are part of the mi-2/NuRD chromatin remodeling complex. Within this complex, MBD2 appears to help target the methylated sequences on DNA, whereas MBD3 contains a loss-of-function mutation in the MBD and facilitates protein-protein interactions. MBD4 is unusual because its most well-studied function is as a DNA repair enzyme, with particular affinity for TpG-CpG mismatches. Lastly, MeCP2 links DNA methylation to repressive chromatin conformation by recruitment of histone deacetylases and histone methyltransferases. MeCP2 may also be involved in the regulation of splicing.

There are numerous post-translational modifications of histones, including acetylation, methylation, phosphorylation, ubiquitination, and biotinylation (144,145). Histone acetylation is particularly dynamic, is controlled by histone acetyltransferases and deacetylases, and is generally associated with active regions of the genome (144). Histone mono-, di-, or tri-methylation is more stable and these marks have been associated with

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either transcriptional activation or repression. Histone modification by phosphorylation, ubiquitination or biotinylation has not been studied as extensively as the acetylation and methylation marks. Phosphorylation appears to play a role in transcriptional activation and chromosome condensation/segregation during mitosis. Ubiquitination and biotinylation are involved in X chromosome inactivation and repression of transposable elements respectively (145). There is a great deal of interplay between the histone modifications, the observation of which led to the development of the “histone code” hypothesis (146) which postulates that combinations of histone modifications could be used to predict the regulatory effect on the gene. However, the concept of epigenetics is continuously evolving and gaining in complexity. More recently discovered epigenetic mechanisms such as the involvement of noncoding RNAs are active areas of research (147).

Evidence from studies of monozygotic twins shows that during early childhood, twins have similar patterns of DNA methylation and histone acetylation, but these similarities are lost in older twins (148). These differences that accumulate over a lifetime might be attributed to environmental factors, including diet, which have been shown to impact epigenetic mechanisms. The effects of methyl-deficient diets have been particularly well-studied in the context of HCC (149). Feeding of methyl-deficient diets to rats resulted in perturbations of folate metabolism, thereby compromising thymidylate and purine synthesis and increasing uracil misincorporation, abasic sites and DNA strand breaks. These phenomena are thought to precede increased DNMT activity, altered expression patterns of DNMTs and MBDs, and abnormal histone modification patterns (150-154). The effects of methyl deficiency on DNA methylation were specific to the liver (153) and were reversible for at least 9 weeks, but not 18 weeks after initiation of the diet by treating with a methyl-replete diet (154), suggesting a possible window for dietary interventions. In a similar fashion, folate deficiency decreases the SAM:SAH ratio and is associated with global and gene-specific DNA hypomethylation in both rodent models and human subjects (74,75,155,156). This effect is reversible with folate supplementation, at least in the short term (155). Folate treatment has also been shown to restore DNA methylation levels to normal in lymphocytes of hemodialysis patients with hyperhomocysteinemia and in livers of aging rats (156,157). Genes of methyl group and homocysteine metabolism have also been implicated in nutrient-gene interactions affecting epigenetic mechanisms (83). In addition to methyl donors, bioactive food components, zinc, selenium, and retinoic acid are all potential modulators of

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epigenetic regulation via effects on methyl group and homocysteine metabolism or epigenetic regulatory proteins (158).

For the studies presented herein, genomic and CpG island DNA methylation, as well as expression of DNMTs and MBDs were used as indicators of epigenetic regulatory processes. DNA methylation status was measured by digestion of DNA with methylation-specific restriction enzymes followed by cytosine extension assay and expression of epigenetic regulatory proteins was determined primarily by real time reverse-transcriptase PCR for relative quantification of mRNA abundance. These methods are well-accepted and have been used previously for the assessment of epigenetic dysregulation (151,153,154), though our laboratory has made minor modifications (detailed in *Materials and Methods* section within each appropriate chapter). These analyses will detect overall changes in the degree of genomic and CpG island DNA methylation, as well as relative expression of the DNMTs and MBDs. DNA methylation patterns may also be assessed at specific genes and we expect that this will be the goal of future studies.

Altered methyl group and homocysteine metabolism including epigenetic regulation

There are numerous factors that can disrupt normal methyl group and homocysteine metabolism. As has been previously discussed herein, these factors include dietary intake and nutritional status, genetic contributions, environmental or pharmacological exposures, and hormonal balance. The majority of the research presented in the remainder of this document is focused on the effects of retinoids and a diabetic condition on methyl group and homocysteine metabolism, including epigenetic regulation of DNA via methylation. Therefore, it is important to review basic concepts related to the function of retinoids and the pathology of diabetes, as well as to examine the current literature describing interactions between retinoids or a diabetic condition and metabolic and epigenetic aberrations.

Retinoids

Retinoids comprise the vitamin A family of compounds including retinyl esters, retinal, retinol, and retinoic acid. Retinal is used in the visual cycle, but for all other

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purposes, retinoic acid is the biologically active form. Retinoic acid is a ligand for the retinoic acid receptor (RAR). The retinoic acid-RAR complex then dimerizes with the retinoid x receptor. This heterodimer recognizes the canonical retinoic acid response elements (RAREs) in the promoter regions of genes, thereby stimulating transcription. The pleiotropic effect of vitamin A may be attributed to the presence of RAREs in the promoter region of genes for several transcription factors which can explain the diverse necessity of vitamin A for cellular differentiation, and optimal reproductive and immune function (159).

Retinoids are widely used in cancer prevention and treatment (159,160), as well as for dermatological disorders such as acne, rosacea, and psoriasis (160). Despite widespread use, there still remain concerns regarding the toxicity of retinoids, thus necessitating further investigation. Retinoic acid is absorbed, transported, and metabolized differently than other forms of vitamin A, such as retinol or carotenoids (161,162). Retinoic acid is rapidly absorbed and is bound by albumin during transport to the liver by the portal vein, rather than via the traditional absorption and transport of fat-soluble vitamins by chylomicrons and entrance to the lymphatic circulation prior to entering the bloodstream. Furthermore, the half-life of all-*trans*-retinoic acid in plasma is <1 hr in both rodents and humans, and retinoic acid is not stored in the liver or extrahepatic tissues (161,163), though it does alter vitamin A metabolism by increasing expression of lecithin:retinol acytransferase, which esterifies retinol to retinyl esters for storage (164,165). Intracellular retinoic acid may be bound to its associated receptors for biological activity, bound by cellular retinoic acid-binding protein, or catabolized (166). The catabolism of retinoic acid to 4-oxoretinoic acid or retinoyl β-glucuronide is catalyzed by cytochrome P450 enzymes, which can be upregulated in response to retinoic acid (164,167). Although this regulatory effect suggests maintenance of vitamin A homeostasis, all-*trans*-retinoic acid is more biologically active and appears to be more toxic than other retinoids (163,166). Hypervitaminosis A presents with nausea, vomiting, fatigue, headache, dermal irritation, skeletal pain, and hepatotoxicity, including elevated serum AST and ALT activity, lipid accumulation, fibrosis, and cirrhosis if left untreated (163,168). Vitamin A toxicity is generally treated simply by halting excess vitamin A intake and addressing conditions which may predispose individuals to toxic effects such as excessive intake of ethanol or of other fat-soluble vitamins, protein malnutrition, and pre-existing liver or renal disease (161,168).

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We have shown that treatment of rats with excess vitamin A at pharmacological doses results in abnormal methyl group and homocysteine metabolism, perturbation of SAM and SAH levels, and DNA hypomethylation (169-171). Rats treated with vitamin A in the form of retinyl palmitate, *cis*-retinoic acid, or all-*trans*-retinoic acid (RA) had elevated hepatic GNMT activity and abundance (47,48,169-171). This effect appears to be specific to the liver, with no changes in GNMT activity and/or abundance observed in the pancreas or kidney (47). The hepatic induction of GNMT was most dramatic in the RA-treated rats, which also exhibited DNA hypomethylation as measured by the SssI methyl-acceptance assay (170). The results of this study have also been replicated in a cell culture model of somatic bovine cells, in which treatment with RA resulted in relative demethylation of DNA (172). The induction of GNMT and DNA hypomethylation were accompanied by increased expression of Oct4, a marker of pluripotency, compared to control cells, thereby indicating deregulation of differentiation. It is also of importance to note that RA treatment in the rat at therapeutic doses was also sufficient for induction of hepatic GNMT, though DNA methylation status was not assessed in that particular study (171).

Mechanistically, the increase in GNMT activity is likely mediated by the effect of retinoids on hepatic folate metabolism. MTHFR activity was suppressed in rats fed a diet high in retinyl acetate or retinyl palmitate, thereby decreasing 5-CH_3-THF levels, presumably relieving the allosteric inhibition of GNMT by 5-CH_3-THF (173). RA treatment has also been shown to increase hepatic MS activity (55,171). However, RA treatment was without effect on PEMT, BHMT, and CBS (48,50,55). The impact of these perturbations on plasma homocysteine and glutathione levels was varied, generally lowering or leaving plasma homocysteine concentrations unchanged. Plasma homocysteine and glutathione levels were unaffected by retinoid treatment alone (169,170), with one report of homocysteine levels markedly decreased by 10 d of high-dose retinoid treatment (171). RA treatment has also been shown to interact with other factors perturbing methyl group metabolism, such as hypo- or hyperthyroidism and diabetes (48,51,55).

There are few reports of the effects of retinoid treatment on DNA methylation status. Retinoic acid is also known to be teratogenic with central nervous system, ear, eye, and craniofacial abnormalities being the most common developmental effects (160,166). In mice, the treatment of dams with RA during pregnancy resulted in DNA hypomethylation of both genomic and CpG island DNA within the palate of the affected offspring (174). In the

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treatment of cancer, retinoids are used as differentiation therapy. This effect has been studied in cancerous and embryonic stem cells models and is proposed to be due in part to epigenetic regulation of imprinted genes and genes for telomerase and cellular regulatory factors (175-177). Interestingly, evidence from acute promyelocytic leukemia cell lines and blasts from patients, it appears that effects on epigenetic regulation may be mediated in part by direct downregulation of the expression of DNMTs by RA (178). The effect of RA therapy on DNA methylation has not been explored in the normal liver in rodents or humans.

Diabetes

Diabetes mellitus affects approximately 12% of the U.S. population and data suggests that the incidence rate will continue to rise in the U.S. and worldwide over the next several years (179). It is estimated that ninety-five percent of cases are type 2, while the remaining 5% are type 1. Though both types of diabetes are characterized by altered glucose metabolism and hyperglycemia, the pathologies of the diseases are different. Type 1 diabetes, often called insulin dependent diabetes mellitus, results from the loss of insulin-producing pancreatic β-cells and consequently, a lack of insulin. Although the mechanisms responsible for β-cell destruction are not entirely clear, in most cases it is proposed to be the result of an autoimmune response that has been associated with exposures to particular viruses and a genetic predisposition (180). Type 1 diabetic patients tend to develop the disease early in life, which is why it has also been called childhood-onset diabetes. Type 2 diabetes had traditionally been thought of as adult-onset diabetes, but is becoming more prevalent in children and adolescents (179). Type 2 diabetes, which is also known as non-insulin dependent diabetes mellitus, is characterized by insulin resistance in which the cells do not respond appropriately to insulin despite hyperinsulinemia (181,182). Insulin resistance is proposed to develop from lipid overload, abnormal lipid metabolism, and chronic inflammation (183). Over time, many type 2 diabetics also develop beta cell dysfunction and impaired insulin secretion. The pathogenesis of type 2 diabetes is believed to originate from a combination of genetic and lifestyle factors. Type 2 diabetes is often clustered with the characteristics of metabolic syndrome, which includes abdominal obesity, elevated serum triglycerides and/or depressed HDL-C, hypertension, and hyperglycemia or insulin resistance (184). Notably, both types of diabetes are also associated with the

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development of secondary complications including macrovascular (i.e. cardiovascular disease, coronary heart disease, stroke) and microvascular (i.e. nephropathy, neuropathy, retinopathy) diseases (179,182). Physiologically, both type 1 and 2 diabetic conditions are also characterized by hyperglycemia and a relative excess of glucocorticoids compared to insulin, whether based on actual concentrations or due to tissue insensitivity.

The rat models of both type 1 and type 2 diabetes used in our studies are well-characterized and closely resemble the condition of human patients. Streptozotocin (STZ) is used to induce a type 1 diabetic state, in part by producing an autoimmune response against the pancreatic beta cells, similar to that observed in human type 1 diabetes (185). Following STZ injection, symptoms of diabetes such as hyperglycemia, hyperphagia, polyuria, and failure to gain or maintain weight manifest within days (186). For our type 2 model, we utilized the Zucker diabetic fatty (ZDF) rat which contains a mutation in the *Lepr* gene, which encodes for the leptin receptor (187). This mutation results in an inability for proper interaction between leptin and leptin receptor, thereby abrogating the suppressive effect of leptin on appetite and insulin secretion. In this way, the pathogenesis of the ZDF rat closely resembles the human type 2 diabetic condition with hyperinsulinemia, progressing to hyperglycemia and eventual beta cell failure. Hyperinsulinemia is observed very early in life, the initial rise in blood glucose occurs between 7 and 8 weeks of age, with a frank diabetic condition presenting by 12 weeks, and beta cell failure occurring between 22 and 42 weeks in male ZDF rats (188). Both animal models are well-established and have been used extensively for the study of diabetes-associated dyslipidemia (189,190), atherosclerosis (191), abnormal vascular response (188,192), nephropathy (186,193,194), retinopathy (195,196), and neuropathy (197,198). However, it may be noted that the STZ-induced model of diabetes exhibits more moderate renal dysfunction than some other models of type 1 diabetes (186), such as the nonobese diabetic mouse, which we have used in subsequent studies.

Perturbations of hepatic methyl group and homocysteine metabolism, as well as the net effect on plasma homocysteine levels, in an early diabetic condition has been well characterized in rodents (**Figure 2**). Upregulation of transmethylation has been observed in both rodent models and human patients. GNMT and PEMT are upregulated by glucocorticoids, STZ- and alloxan-induced type 1 diabetes (32,48-50). Treatment of rats or

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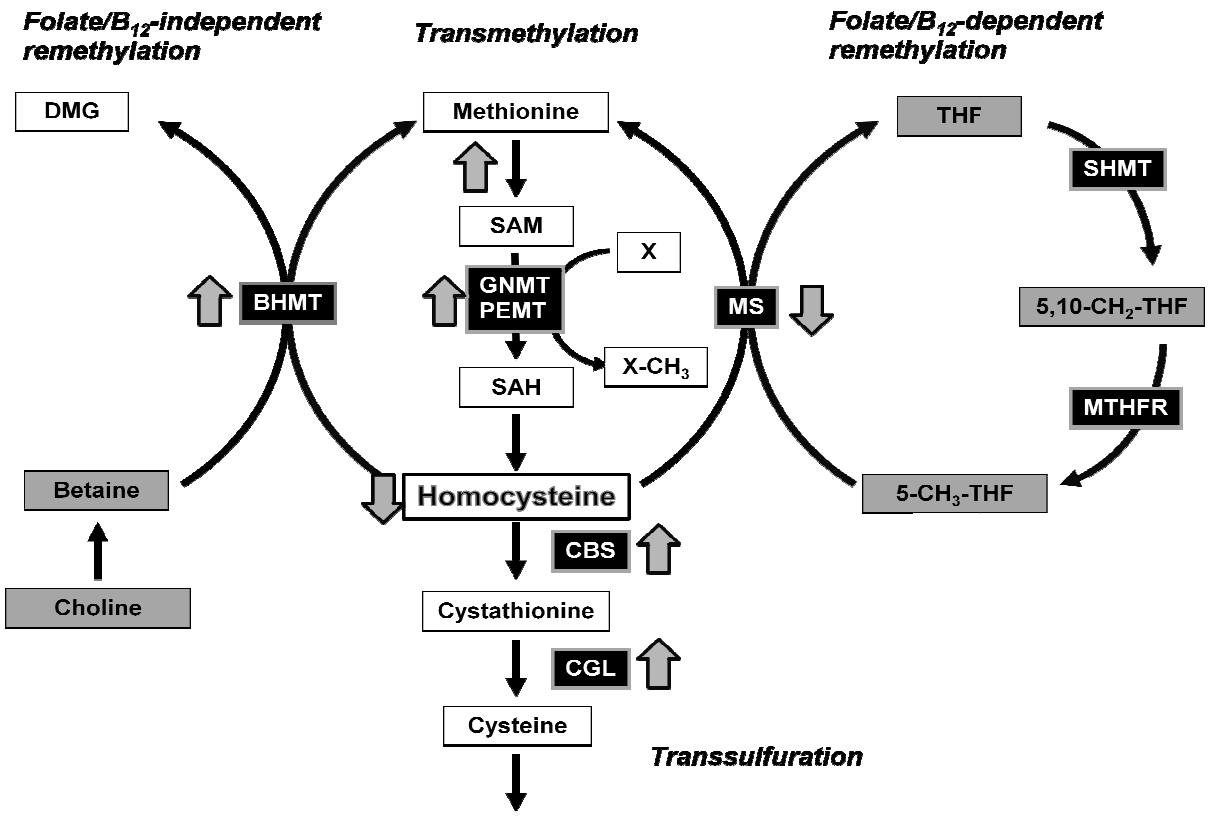

Figure 2. Plasma homocysteine levels and activity of hepatic enzymes of methyl group and homocysteine metabolism in diabetes.

Abbreviations: 5-CH_3-THF, 5-methyltetrahydrofolate; 5,10-CH_2-THF, 5,10-methylenetetrahydrofolate; BHMT, betaine-homocystiene *S*-methyltransferase; CBS, cystationine β-synthase; CGL, cystathionine γ-lyase; DMG, dimethylglycine; MTHFR, methylenetetrathydrofolate reducttase; MTs, methyltrasnferases; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate; SHMT, serine hydroxymethyltransferase; X, methyl acceptor

H4IIE rat hepatoma cells with the glucocorticoid dexamethasone also induced GNMT expression and activity (51). Administration of insulin normalized induction of GNMT and PEMT in the STZ-diabetic rat (49,50). Increased PEMT activity by STZ was associated with a decreased ratio of PE to PC in the liver (50). GNMT was also upregulated in the ZDF rat, but PEMT expression in this model is unknown (52).

The remethylation and transsulfuration pathways are also affected in a diabetic condition. There have been variable responses of MS and MTHFR to a diabetic condition as evidenced by reports of increased activity in response to glucocorticoid treatment (60) or pre-diabetes (52), decreased activity in type 1 diabetes (49), and no change in STZ-diabetes

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(199) and early type 2 diabetes (52). In contrast, the effect of a diabetic condition on BHMT is consistent and well-characterized. BHMT mRNA expression and/or activity were increased in diabetic rats and in hepatoma cells treated with glucocorticoids (48-50,52,200); this effect was reversed or attenuated with treatment by insulin in the STZ-induced diabetic model (200). Hepatic CBS and CGL activities were increased in both glucagon-treated, as well as STZ-diabetic and ZDF rats (48,52,126,201). CBS mRNA abundance was also increased in glucocorticoid-treated hepatoma cells (201). Insulin abrogated the increases in CBS activity (201,126). The effects of STZ-diabetes and insulin treatment were also observed at the transcriptional level as evidenced by similar changes in CBS mRNA (201).

There have been very few studies of methyl group and homocysteine metabolism in the diabetic kidney. House et al (126) reported a lack of effect of either glucagon treatment or STZ-diabetes, whereas Jacobs et al (199) reported that renal MS and MTHFR activities were suppressed by a type 1 diabetic condition. Methyl group and homocysteine metabolism have not be investigated in the kidney of the ZDF rat. Thus, all changes discussed thus far were observed in the liver and in an early diabetic condition (i.e. within 10 d of onset). The net effect of these perturbations results in lowered plasma homocysteine levels (48-52). Early in the course of type 1 diabetes, diabetic patients also have been found to have lower fasting and post-methionine load plasma homocysteine concentrations relative to healthy controls (202). However, in advanced diabetes it has been shown that MTHFR activity was suppressed in lymphocytes of diabetic patients (128) and both hepatic transmethylation and transsulfuration flux were decreased in diabetic nephropathy (129). This occurrence of metabolic disturbance in advanced diabetes, concurrent with the development of hyperhomocysteinemia in diabetic nephropathy (128,203) suggests the need to study the chronic diabetic condition, with particular attention to the influence of both the liver and the kidney.

Recently, diabetes has also been associated with alterations of epigenetic mechanisms, specifically histone modifications. These studies of both cell culture and mouse models of hyperglycemia or diabetes have identified pathways or categories of genes with altered patterns of histone methylation. The targets of this regulation include proteins involved in signal transduction, oxidative stress, immune function, and inflammatory pathways (204-208). These effects were maintained even after the restoration of normoglycemia, suggesting a “metabolic memory” (204-206) which should be characteristic

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of true epigenetic regulation. The alterations of histone modifications were also cell-type specific (207,208). This highlights the need for study of epigenetic regulation in the tissue of interest whenever possible. Efforts should also be made to profile DNA methylation status of genome to complement this knowledge of histone modifications.

Our long-range goal is to understand how diabetes-mediated alterations in metabolic processes may serve as a link to the numerous complications associated with diabetes, and how dietary intervention can prevent these adverse effects. The focus of the studies presented here was on perturbations of key enzymes and metabolites of methyl group and homocysteine metabolism that occur in both type 1 and type 2 diabetes, and how these changes may be linked to alterations of epigenetic regulation. To date, most studies have assessed only hepatic metabolism in the early diabetic condition and epigenetic regulation in diabetes has not been fully addressed within tissues of interest. This includes the liver, kidney, and heart as tissues which contain the full complement of methyl group and homocysteine metabolic pathways and/or are affected by the secondary pathologies of diabetes. Lack of such knowledge is an important problem, because understanding the metabolic link between these processes is essential for establishing dietary recommendations for preventing metabolic and epigenetic aberrations which may be related to secondary complications of diabetes, a major focal point for future research.

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CHAPTER 3: Long-term, thrice-weekly supplementation with retinoic acid alters methyl group metabolism, but does not induce changes in DNA methylation status in rat liver

A manuscript to be submitted to Hepatology.

Kelly T. Williams and Kevin L. Schalinske

Abstract

S-adenosyl methionine-dependent transmethylation reactions are important in many biosynthetic and regulatory processes such that compromised methyl group availability results in a number of pathologies. For example, global DNA hypomethylation is associated with genomic instability and overexpression of genes. Glycine *N*-methyltransferase is a key protein in the regulation of methyl group supply and utilization; thus, it is important to understand how nutritional and/or hormonal factors influence GNMT. Previously it has been demonstrated that administration of all-*trans*-retinoic acid (RA) rapidly induces glycine *N*-methyltransferase and global DNA hypomethylation during short-term (7-d) studies. The aim of this study was to investigate the long-term effects of RA administration on GNMT expression, DNA methylation, and hepatotoxicity. Rats were treated orally with either 0, 5, or 30 μmol RA/kg body weight three times per week for a total of 2, 4, 8, 16, or 24 wk. Treatment with either dose of RA induced GNMT activity and abundance at 2 wk, and this increase was observed at all timepoints in the study. Serum activities of alanine aminotransferase and aspartate aminotransferase were not affected by RA treatment. Interestingly, RA treatment resulted in significantly decreased hematocrit levels, as well as lowered plasma glutathione concentrations. Chronic administration of RA results in a sustained increase in GNMT activity and protein abundance with little indication of hepatotoxicity and no effect on DNA methylation status. However, chronic administration of retinoic acid, even at therapeutic levels, can adversely impact hematopoiesis and antioxidant defense.

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Introduction

Folate-dependent one-carbon metabolism is central to a number of biochemical processes including the biosynthesis of nucleic acids and the regeneration of methionine from homocysteine by methionine synthase (MS) (1). The methyl donor for the MS reaction is 5-methyltetrahydrofolate (5-CH_3-THF), which is synthesized by methylenetetrahydrofolate reductase (MTHFR) from 5,10-methylenetetrahydrofolate (5,10-CH_2-THF). The products of the MS reaction are methionine and tetrahydrofolate, which can be recycled back into the folate pool. Methionine can be activated to *S*-adenosylmethionine (SAM), which serves as a methyl group donor for more than 100 different SAM-dependent transmethylation reactions (2,3). Methyltransferases catalyze the transfer of a methyl group from SAM to a methyl acceptor molecule, which may include proteins, lipids, nucleic acids or other small molecules, thereby generating a methylated product and *S*-adenosylhomocysteine (SAH), which can subsequently be metabolized to homocysteine. Most SAM-dependent methyltransferases are inhibited by SAH, therefore the SAM:SAH ratio is purported to be an indicator of transmethylation potential.

Glycine *N*-methyltransferase (GNMT) is highly abundant in liver (1-3% of cytosolic protein) and is a proposed regulator of hepatic methyl group supply and utilization (2,4). The reaction catalyzed by GNMT involves the transfer of a methyl group from SAM to glycine, thereby forming sarcosine, a product for which the physiological function is unclear. The regulation of GNMT is unique in that it is inhibited by SAH to a lesser extent than most other methyltransferases and is intricately tied to one-carbon folate metabolism (**Figure 1**). GNMT is allosterically inhibited by 5-CH_3-THF (5), whereas MTHFR activity is suppressed by SAM (6). Therefore, when methyl group supply is high, SAM concentrations are elevated and MTHFR activity is inhibited, decreasing the synthesis of 5-CH_3-THF and relieving inhibition of GNMT. Increased GNMT activity would be expected to decrease SAM levels, thereby restoring the SAM:SAH ratio. Conversely, when methyl supply is low, SAM levels would also be decreased, relieving the inhibition of MTHFR, increasing 5-CH_3-THF concentrations and inhibiting GNMT, thereby conserving methyl groups. Taken together with recent reports that the SAM:SAH ratio is elevated up to 100-fold in two newly developed GNMT knockout mouse models (7,8), there is strong evidence for the role of GNMT in the regulation of transmethylation potential. Given this critical regulatory role, it is

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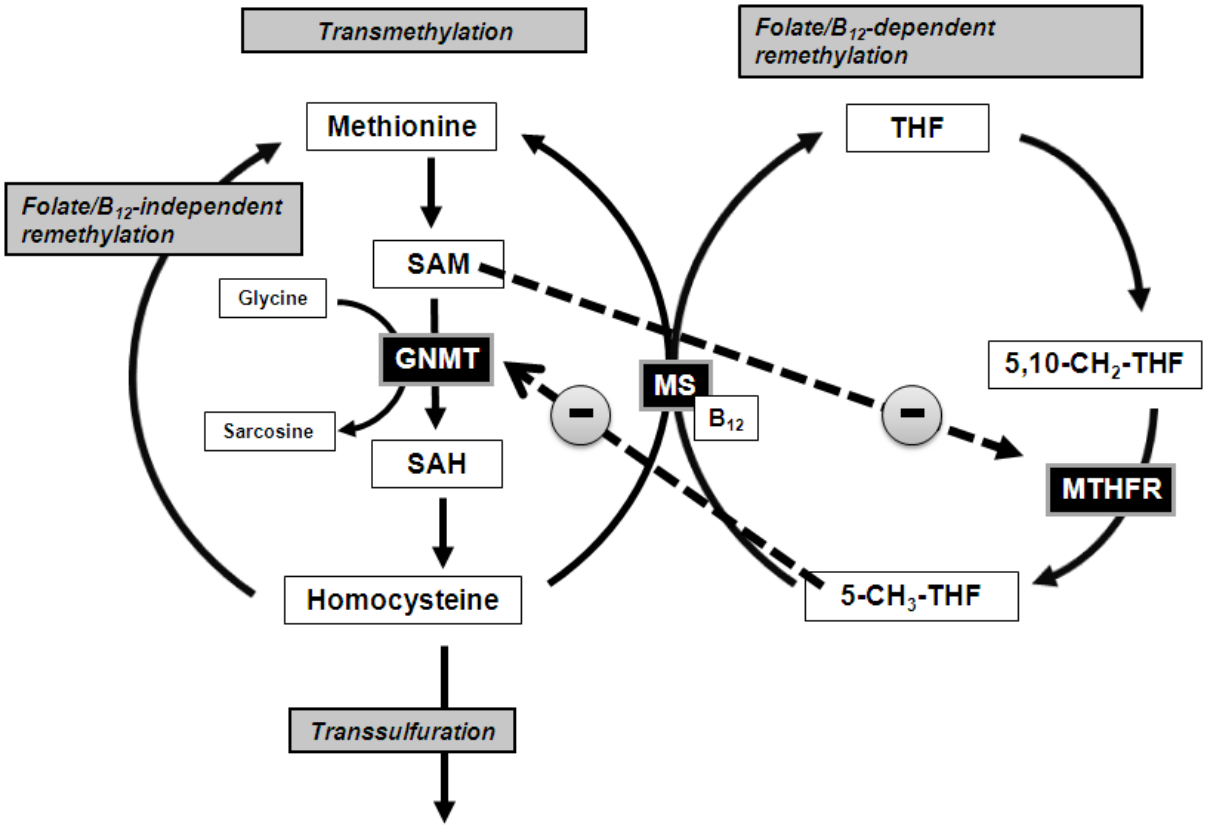

Figure 1. Regulation of folate, methyl group, and homocysteine metabolism.

Abbreviations: 5-CH_3-THF, 5-methyltetrahydrofolate; 5,10-CH_2-THF, 5,10-methylenetetrahydrofolate; GNMT, glycine *N*-methyltransferase; MTHFR, methylenetetrahydrofolate reducttase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate;

important to gain a greater understanding of how nutritional or pharmacological factors may influence GNMT expression and activity.

Retinoid compounds are widely used as therapies for dermatological conditions and in cancer prevention and treatment (9,10). It has previously been demonstrated that large daily doses of retinoid compounds can alter folate one-carbon and methyl group metabolism, in part by the induction of GNMT. We have shown that GNMT activity and protein abundance increases in response to treatment with retinyl palmitate, 9-*cis*-retinoic acid or all-*trans*-retinoic acid (RA) (11-13). RA was the most potent inducer of GNMT and

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also resulted in significant DNA hypomethylation during short-term studies (7-d), suggesting that the induction of GNMT compromises methyl group availability for other SAM-dependent transmethylation reactions (13). It has long been known that the reduction of methyl group availability by chronic feeding of a methyl-deficient diet results in global and gene-specific DNA hypomethylation, which is associated with genomic instability and overexpression of genes, specifically the proto-oncogenes c-*myc,* c-*fos*, and c-*ras* (14-16). The long-term adverse effects of a methyl deficient diet are an independent cause of liver fibrosis, cirrhosis, and hepatocellular carcinoma. Hepatotoxicity is also a potential side effect of retinoids, with increased serum ALT and AST activity reported in up to 33% of patients (10). Although the impact of short-term retinoid treatment on methyl group metabolism is well-characterized, data are lacking for effects of retinoid administration after several months, which is the usual duration of therapeutic treatments. Therefore, the aim of the current study was to investigate the long-term effects of RA administration on GNMT expression, DNA methylation and hepatotoxicity

Materials and Methods

Chemicals and reagents Reagents were obtained as follows: 2,4-dinitrophenylhydrazine, RA, Sigma-Aldrich Chemical Co. (St Louis, MO); Coomassie Plus Protein Reagent, Pierce Chemical; enhanced chemiluminescence Western blotting detection reagents, Amersham Biosciences; goat anti-mouse horseradish peroxidase secondary antibody Southern Biotechnology (Birmingham, AL); phenylmethylsulfonyl fluoride (PMSF), Calbiochem; S-adenosyl-L-[*methyl*-^{3}H] methionine, New England Nuclear (Boston, MA). GNMT antibodies were provided by Yi-Ming Chen, National Yang-Ming University, Taipei, Taiwan (17). All other chemicals and reagents were of analytical grade.

Animals All animal protocols were approved by and conducted in accordance with the guidelines set forth by the Iowa State University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (50-75 g at onset) were housed in individual cages with a 12:12 hr light:dark cycle, and given free access to water and semi-purified diet as previously described (11). Rats were randomly assigned to a treatment dose and duration

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(n= 5-6 per group, 72 animals total). The treatment regimen consisted of 0, 5, or 30 µmol RA/kg body weight (control, low RA, high RA) administered orally in corn oil vehicle three times per week for a total of 2, 4, 8, 16, or 24 wk. Treatment doses were chosen based on the normal dosages for retinoid therapies and based on previous dose-response studies demonstrating that the greatest induction of GNMT was with a dose of 30 µmol/kg body weight (18). The 5 µmol/kg body weight dose is equivalent to 1.5 mg/kg body weight; treatment for dermatological conditions is usually prescribed at 0.5-2 mg/kg body weight/d for 6 months (19). Treatment protocol was designed to minimize dermatological irritation.

All treatments were initiated immediately after an acclimation period and rats were euthanized at the end of the each treatment period. Animals were anesthetized by intraperitoneal injection with ketamine and xylazine (90 and 10 mg/kg respectively), heparinized whole blood samples were collected by cardiac puncture, and livers were rapidly excised. An aliquot of whole blood was collected in hematocrit capillary tubes, sealed, and percent hematocrit was determined after centrifugation for 5 min in an Autocrit Ultra 3 Microhematocrit Centrifuge (BD Diagnostics, Franklin Lakes, NJ). A portion of the liver was immediately homogenized and cytosolic extracts prepared as previously described (20,21); the remainder was snap frozen in liquid nitrogen and subsequently stored at -70℃.

GNMT activity and abundance GNMT activity was determined based on the method of Cook and Wagner (22) with minor modifications (11). Briefly, 250 µg protein was added to a reaction mixture of 0.2 mol/L Tris buffer (pH 9.0), 5 mmol/L dithiothreitol, 2 mmol/L glycine, and 0.2 mmol/L *S*-adenosyl-*L*-[*methyl*-^{3}H]methionine and incubated at 25℃ for 30 min. Reaction was stopped by addition of 10% trichloroacetic acid and unreacted SAM was removed by addition of activated charcoal and centrifugation. Aliquots of supernatant were used for liquid scintillation counting. GNMT abundance was assessed using immunoblotting and subsequent chemiluminescent detection as described previously (20). SigmaGel Software (SPSS, Chicago, IL) was used for densitometric analysis of relative abundance.

Plasma homocysteine and glutathione Plasma samples were derivatized for determination of plasma homocysteine and glutathione as described by Ubbink et al (23)

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with minor modifications (24). Homocysteine and glutathione were analyzed by HPLC with fluorometric detection by injecting 100 µL of sample onto a µBondapak C_{18} Radial-Pak column (Waters, Milford, MA) using a mobile phase of 40 mL/L acetonitrile in 0.1 mol/L potassium phosphate buffer (pH 2.1). *N*-acetylcysteine (1 mmol/L) was used as an internal standard.

DNA methylation Genomic DNA was isolated from snap frozen tissue using a commercial kit (cat # A1125, Promega). Global and CpG island DNA methylation status was determined by the method of Progribny et al (25) and as described previously (21). Briefly, 1 µg DNA was digested with the methylation-sensitive restriction enzymes *HpaII* and *BssHII* – for assessment of global and CpG island DNA methylation respectively, followed by cytosine extension assay with Amplitaq DNA Polymerase (Applied Biosystems), and [^{3}H]-dCTP. After samples were applied to Whatman anion exchange paper, washed in 0.5 mol/L sodium phosphate buffer, and dried, liquid scintillation counting was utilized for determination of ^{3}H incorporation.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity
Plasma levels of ALT and AST activity were determined using the Reitman-Frankel method (26) with minor modifications. Briefly, 100 µL of plasma was added to 500 µL substrate mix and incubated at 37˚C for 30 min or 1 hr for ALT and AST assays, respectively. The substrate mix consisted of 0.1 mol/L sodium-potassium phosphate buffer (pH 7.4), 2 mmol/L α-ketoglutarate, and 200 mmol/L alanine or aspartate for assessment of ALT or AST respectively. Following incubation, 500 µL of a color reagent containing 0.1 mol/L 2,4-dinitrophenylhydrazine in 1 mol/L HCl was added to all samples, which were then incubated at room temperature for 20 min. Absorbance was measured on a spectrophotometer at 510 nm and compared to a standard curve for determination of ALT and AST activity.

Statistical analysis For each timepoint, data for each treatment group were subjected to a one-way ANOVA followed by Fisher's least significant difference test for multiple comparisons (SigmaStat, SPSS, Chicago, IL). When equal variance or normality tests

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failed, the ANOVA on ranks procedure was utilized. Data from all timepoints were analyzed by two-way ANOVA followed by Fisher's least significant difference test for multiple comparisons to test for effects of time, RA treatment, or time x treatment interactions. Differences were considered significant at $P < 0.05$; trends were noted at $0.05 < P < 0.1$.

Results

Induction of GNMT by long term thrice-weekly RA treatment

Elevation of GNMT activity by administration of RA was observed at all measured timepoints, with the exception of 4 wk (**Figure 2A**). The increases in GNMT activity were generally associated with increases in GNMT abundance, which were observed at all timepoints (**Figure 2B**). Consistent with our previous short-term studies (18), RA administration resulted in the elevations of GNMT activity and abundance in a dose-dependent manner. Moreover, this effect was also observed at both early and late timepoints in this study of RA treatment.

RA treatment had no effect on DNA methylation status

As measured by the method of Pogribny et al (25), an increase in [^{3}H]-dCTP incorporation into DNA digested by methylation-specific restriction enzymes indicates hypomethylation, whereas a decrease indicates hypermethylation. However, there were no observed differences in [^{3}H]-dCTP incorporation between treatment groups at 2 or 24 wk whether the DNA was digested with *HpaII* or *BssHII* (**Table 1**). This signifies that there were no overall changes in global or CpG island DNA methylation status.

Indicators of hepatotoxicity were minimally affected by RA treatment

RA treatment had no effect on rat weight or weight gain between groups at each respective timepoint (**Table 2**). Likewise, there was no evidence of RA-induced hepatomegaly based on a lack of effect of RA administration on relative liver weight (**Table 2**). However, when all data was subjected to a two way ANOVA, in addition to the

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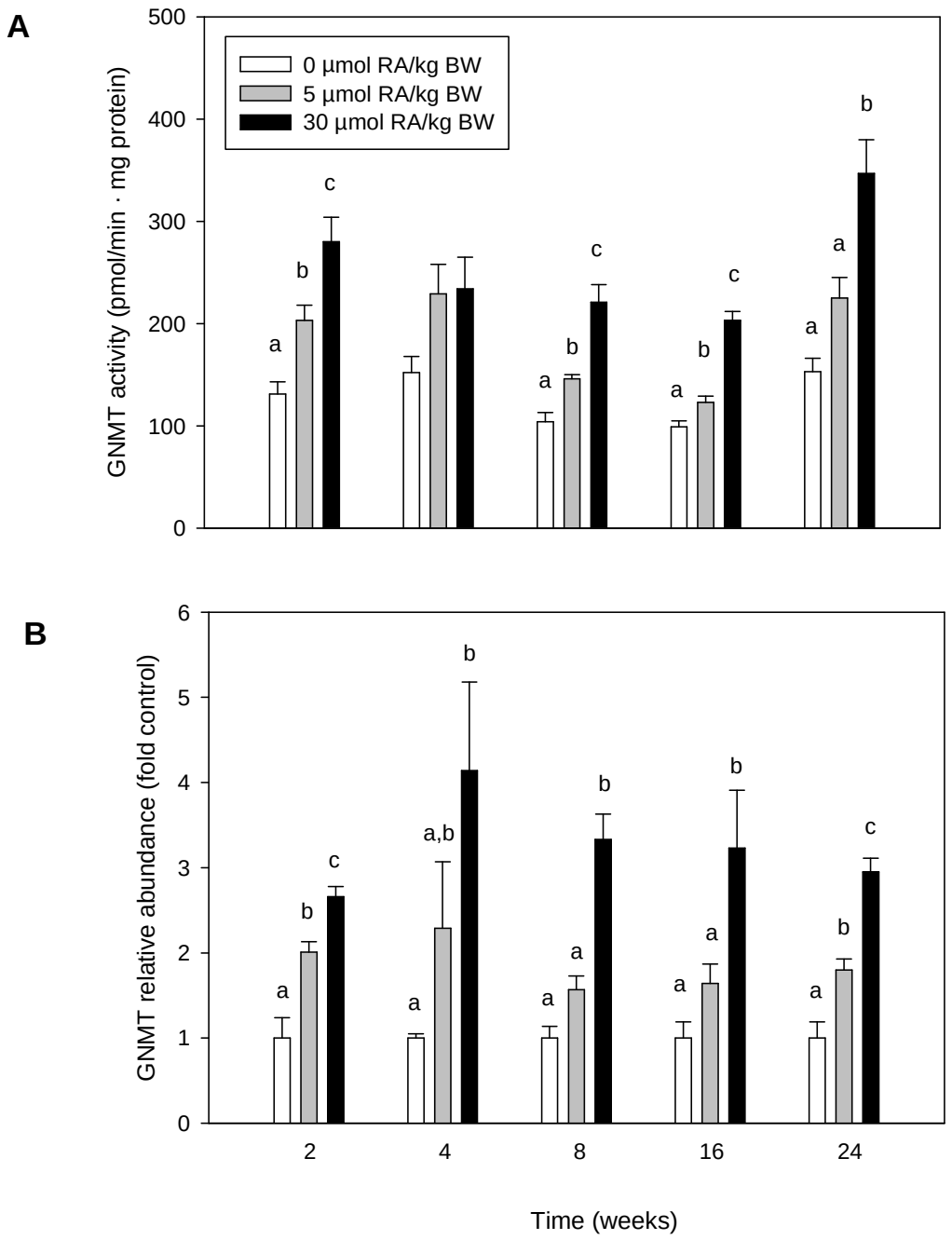

Figure 2. RA treatment of rats at 5 or 30 µmol/kg body weight increases GNMT activity and/or abundance in a dose-dependent manner. (A) Hepatic GNMT activity levels and (B) hepatic GNMT protein levels relative to the control, data are means ± SEM, different letters denote a significant difference between groups within that timepoint, $p < 0.05$.

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Table 1. Global and CpG island DNA methylation status were unaffected by long-term intermittent RA treatment.

Time (wk)	2			24		
Treatment (µmol RA/kg BW)	0	5	30	0	5	30
Global methylation *^{3}H-dCTP incorporation (DPM/µg DNA)*	8000 ± 994	7418 ± 426	7019 ± 419	7706 ± 913	6573 ± 763	6294 ± 597
CpG island methylation *^{3}H-dCTP incorporation (DPM/µg DNA)*	435 ± 39	581 ± 74	426 ± 66	680 ± 83	673 ± 71	737 ± 61

Data are means ± SEM (n = 5-6). Data were compared between groups within each timepoint and assay.

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Table 2. Body weight, weight gain, and relative liver weight were unaffected by RA treatment

	Body weight (g)				Cumulative weight gain (g)				Relative liver weight (% body weight)			
Treatment (μmol RA/kg body weight)	0	5	30	p-value	0	5	30	p-value	0	5	30	p-value
Time (wk)												
2	165 ± 4	174 ± 8	171 ± 6	0.576	77 ± 2	82 ± 6	84 ± 6	0.557	4.9 ± 0.2	5.4 ± 0.1	5.2 ± 0.2	0.135
4	255 ± 10	238 ± 6	242 ± 8	0.344	167 ± 9	151 ± 6	154 ± 9.5	0.325	4.2 ± 0.1	4.3 ± 0.2	4.1 ± 0.1	0.728
8	351 ± 7	330 ± 10	321 ± 16	0.199	264 ± 6	244 ± 8	235 ± 16	0.370	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	0.988
16	446 ± 12	437 ± 20	405 ± 12	0.170	357 ± 12	346 ± 18	318 ± 13	0.163	2.9 ± 0.1	2.9 ± 0.1	3.0 ± 0.1	0.429
24	471 ± 16	482 ± 7	463 ± 18	0.668	385 ± 14	388 ± 12	380 ± 18	0.935	2.7 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	0.146

Data are means ± SEM (n = 6).

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significant effects of time ($p < 0.001$), as would be expected due to growth of the animals, there were also slight trends towards decreased body weight ($p = 0.062$) and weight gain ($p = 0.098$), as well as increased relative liver weight ($p = 0.097$) associated with RA treatment.

Plasma ALT and AST activity levels at 24 wk were also used as biomarkers of hepatotoxicity. Activities of these enzymes are high in liver, but normally low in plasma, therefore ALT and AST activities are only high in plasma when damage to the liver results in release of the enzymes into the circulation. There were no significant differences observed in plasma ALT or AST activity at 24 wk (**Table 3**), indicating a lack of hepatotoxicity.

Long term RA treatment decreased plasma glutathione concentrations.

RA treatment did not have a significant effect on plasma homocysteine levels at any of the individual timepoints assessed. However, two-way ANOVA revealed a trend towards a decrease in homocysteine with RA treatment ($p = 0.073$, representative data from 24 wk shown in **Table 3**). At 24 wk, glutathione levels were found to be decreased by 28% in animals receiving the high RA treatment compared to the control group ($p = 0.013$). The plasma glutathione concentration was also 11% lower in low RA vs. controls, though this difference did not attain significance ($p = 0.103$). There were no differences in glutathione levels between treatment groups detected at earlier timepoints or by two-way ANOVA analysis (data not shown).

Hematocrit levels are decreased by long-term RA administration

Given the well-established interaction between vitamin A and iron metabolism, as well as the role of vitamin A status on cellular differentiation, hematocrit levels were determined as a measure that would be sensitive to either of these effects. There were no changes observed in hematocrit levels at 2 wk, but beginning at 4 wk hematocrit levels were lower in RA-treated rats compared to control animals (**Figure 3**). In general, this decrease in hematocrit was observed in all rats receiving RA, and two-way ANOVA revealed a significant effects of RA, time, and a treatment by time interaction ($p < 0.001$).

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Table 3. Plasma homocysteine concentrations, as well as ALT and AST activity levels were unchanged, whereas glutathione concentrations were decreased by 24 wk of RA treatment

Treatment (µmol RA/kg BW)	**ALT Activity** (U/L)	**AST Activity** (U/L)	**Homocysteine** (µmol/L)	**Glutathione** (µmol/L)
0	14.1 ± 1.8	75.45 ± 8.24	9.27 ± 1.29	12.50 ± 0.75[a]
5	13.6 ± 1.0	69.59 ± 13.33	8.86 ± 0.85	11.2 ± 1.10[a,b]
30	12.2 ± 0.7	85.07 ± 10.17	7.72 ± 0.51	8.96 ± 0.77[b]

Data are means ± SEM (n = 5-6). Different superscript letters within the column indicate a significant difference, $p < 0.05$.

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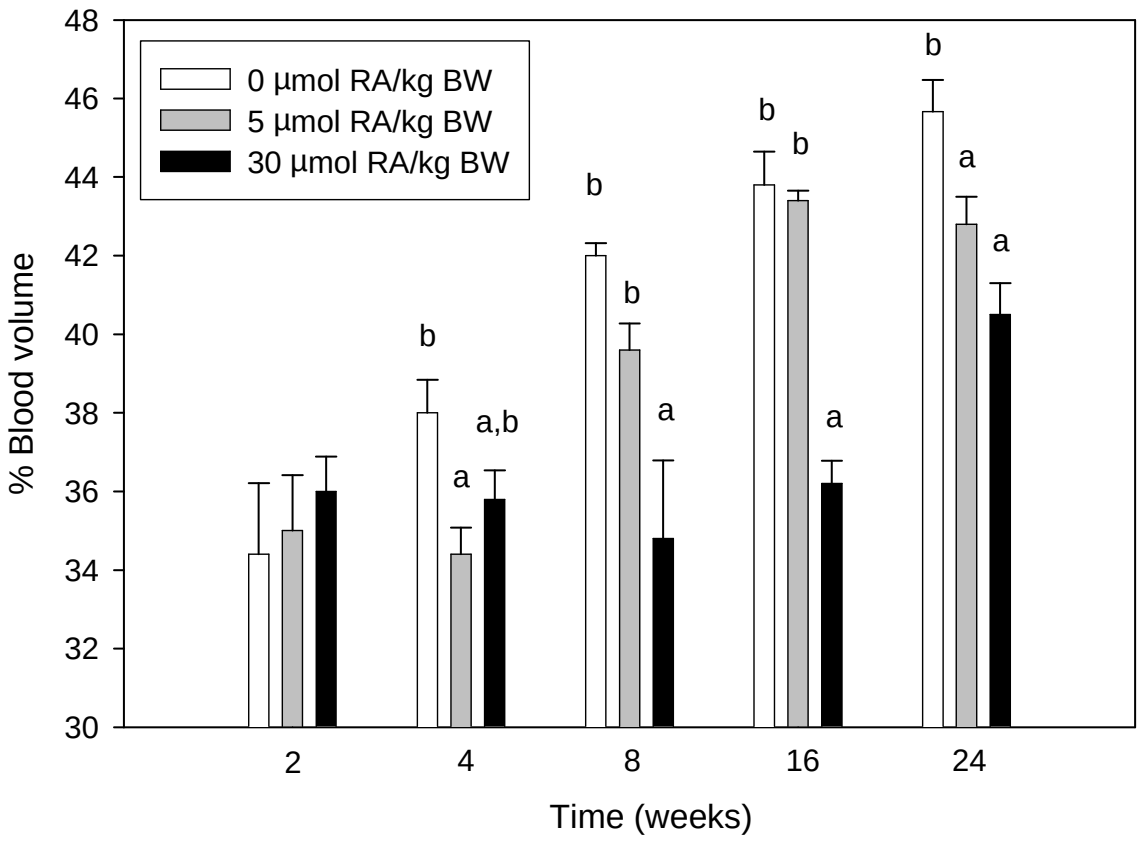

Figure 3. Hematocrit levels are decreased by RA treatment. Data are means ± SEM (n = 5-6). Different letters within a time indicate a significant difference between group means, $p < 0.05$.

Discussion

Homeostasis of methyl group and one-carbon metabolism are essential for many processes fundamental to health due to involvement in cellular growth and differentiation, as well as methylation of critical cell components. Although short-term effects of RA treatment on hepatic methyl group and homocysteine metabolism have been established, chronic effects have yet to be fully investigated. In this study, we found that long-term thrice-weekly RA administration resulted in sustained induction of hepatic GNMT activity, but was without effect on DNA methylation status. There were minimal to no signs of hepatotoxicity, but

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decreased plasma glutathione and markedly lower hematocrit in RA-treated rats suggests the potential for adverse effects systemically due to decreased antioxidant defense or on specific extrahepatic tissues, such as bone.

We have previously demonstrated that the induction of GNMT by RA is rapid, significant after just one treatment, and dose-dependent (18). The induction of GNMT is also consistently characterized by elevations in both activity and protein abundance (11-13). Similar to these short-term studies, we have demonstrated that GNMT activity and/or abundance in rats treated with RA at all timepoints from two to 24 wk. The increase in GNMT may be due in part to the effects of RA on folate one-carbon metabolism. Rats fed a diet high in retinyl acetate or retinyl palmitate or treated with RA have suppressed MTHFR activity and/or elevated MS activity (15,24,27). Either of these alterations, or both in concert, would be expected to lower 5-CH_3-THF concentrations, which was measured and observed in rats fed high-vitamin A diets (27). GNMT activity can be enhanced by phosphorylation of the protein, but when 5-CH_3-THF is bound to GNMT, this phosphorylation was shown to be inhibited *in vitro* (5). Therefore, with lower intracellular levels of 5-CH_3-THF in the liver, the allosteric regulation of 5-CH_3-THF would be relieved, GNMT might be more easily phosphorylated and specific activity might increase. Perturbation of folate status and homocysteine concentrations have been documented in two studies of humans treated with retinoids. Patients treated with 13-*cis*-retionic acid (0.5 mg/kg body weight) for 45 d had elevated plasma homocysteine (28), whereas Chanson et al (29) found no change in plasma homocysteine, but folate metabolism was perturbed such that 5-CH_3-THF levels decreased by ~15-20%.

In addition to the potential role of altered folate metabolism, transcriptional and/or translational control of GNMT is suggested by the strong correlation between GNMT protein abundance and activity as was found here ($r = 0.511$, $p < 0.001$) and in a previous study (13). Although approximately half the change in GNMT activity can be explained by altered transcriptional and/or translational control of GNMT by RA administration, the mechanism by which this occurs is not fully understood. A likely explanation may be found in the recent analysis of the promoter region of GNMT which identified several putative RAR and RXR binding sites within the 1800 kb upstream of the transcriptional start site (30). Furthermore, a binding site for Sp1 was also identified and Sp1 has been implicated in RA-induced expression of other proteins in several extrahepatic tissues (31-33). Immunoprecipitation

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for RAR or RXR followed by immunoblotting for RAR and Sp1 has demonstrated that RAR/RXR can interact directly with Sp1 to stimulate transcription (31). However, the induction of GNMT by retinoids has been shown to be tissue-specific (13) and these interactions between RAR/RXR and their putative binding sites within the GNMT promoter or Sp1 have not been described in the liver. Rather, when HepG2 cells, a rat hepatoma cell line, were used to characterize the interactions at the human MRP3 gene promoter, RAR/RXR was shown to decrease Sp1 binding in a dose-dependent manner (34). Notably, the promoter for this gene does not appear to contain either a RARE or atypical RARE, so decreased Sp1 binding may not be the effect observed within a promoter with a RARE site in liver. Additional characterization of the GNMT promoter and its interactions with transcription factors presents a promising direction for future investigations into the regulation of GNMT.

Despite the long-term upregulation of GNMT, the level of global and CpG island DNA methylation and plasma homocysteine levels were maintained. We hypothesize this might be due to the intermittent nature of the treatment regimen or that other transmethylation reactions might be compromised in favor of maintenance of DNA methylation patterns. Retinoic acid has unique characteristics of absorption, transport, and metabolism relative to other forms of vitamin A, such as retinol or carotenoids (35,36). Retinoic acid is rapidly absorbed and travels to the liver via the portal vein and bound by albumin, rather than by absorption and transport in chylomicrons and entrance to circulation after lymphatic circulation, as for other forms of vitamin A. Additionally, the half-life of all-*trans*-retinoic acid in plasma is <1 hr in both rodents and humans, and it is not stored in neither liver nor extrahepatic tissues (35,37). Given the observed kinetics of retinoic acid, it is possible that daily RA treatment is necessary for alteration of DNA methylation status and also suggests the possible influence of other regulatory mechanisms in addition to the upregulation of GNMT. It is also possible that there were specific changes in DNA methylation patterns that were not measurable by this assay, which only assesses total changes in methylation status. Interestingly, since our initial report of RA-induced hypomethylation, studies have been performed *in vitro* with the aim of identifying agents for reprogramming of cells for cloning. Eilertsen et al (38) report that in bovine somatic cells, treatment with RA (100 nmol/L) induced GNMT activity, resulting in a 25% decrease in 5-methylcytosine content of DNA and upregulation of Oct4, a biomarker of pluripotency. Although this study was preliminary in nature, it is interesting to note the dramatic effects on cellular phenotype

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associated with RA-induced upregulation of GNMT expression and DNA hypomethylation. Genomic and CpG island DNA hypomethylation have also been observed in the tissue of cleft palates induced by maternal RA treatment of mice during pregnancy (39). Unfortunately, there appears to be little to no data characterizing the effect of RA on DNA methylation or the complete complement of methyl group and homocysteine pathways in humans. Given the evidence of altered folate and homocysteine metabolism in humans (28,29), this may be a worthy avenue of investigation.

In this study, there were few signs of hepatotoxicity as evidenced by a lack of effect on plasma ALT and AST activities, and very moderate trends towards decreased body weight, weight gain, and increased relative liver weight. Retinoid treatment in dermatological patients may be accompanied by transient increases in ALT or AST that are normally reversed by ending treatment (40). In this experiment, ALT and AST activity was only measured at 16 and 24 wk (16 wk data not shown) and although there were no significant differences between groups at either time, it's possible that a transient increase was missed. Our results are also consistent with the results of Hotchkiss et al (41), who also found no change in plasma ALT and AST activity in rats given 10 or 15 mg RA/kg body weight – a larger dose than that used here, more similar to that prescribed to leukemia patients - for 10 or 15 wk. These results indicate minimal risk of hepatotoxicity; however chronic RA-treatment was not without negative effects.

Hematocrit was significantly decreased in response to both low and high RA treatment. While the control animals' hematocrit levels increased, as would be expected for a growing rat (42), hematocrit levels did not increase at a similar pace in RA-treated animals, suggesting compromised hematopoiesis. Although vitamin A treatment is often used for the prevention and treatment of anemia (36), there are also clinical, experimental, and epidemiological data that retinoic acid can negatively affect bone and/or blood cell formation. In a case study of an infant suffering from vitamin A intoxication, the bone marrow aspirant was found to contain vacuolated or binucleated erythroblasts (43). A follow up *in vitro* study of primary bone marrow mesenchymal stem cells and K-562 cells (multipotent, hematopoietic) found that exposure to 20-80 μmol/L RA resulted in the inhibition of cell proliferation (43). Alternatively, bone marrow diameter and area were decreased in both male and female rats given 15 mg RA/kg body weight for a treatment period of 15 weeks (41). However, given the larger dosage used in this study and the lack

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of effect in rats given a lower dose of RA or 13-*cis*-retinoic acid, it seems unlikely that reduced marrow content could account for the differences in hematocrit observed in our investigations. The epidemiological data tells a slightly different story. The results of four large epidemiological studies regarding the effect of vitamin A on bone suggests that intake as low as twice the RDA could be associated with increased risk of osteoporosis and risk of fracture (36). Although others have not always shown a similar effect, there are several other potential side effects that warrant consideration in a risk-benefit analysis of treatment with retinoids. In addition to the commonly reported side effects such as skin and eye irritation, retinoids are well known to be teratogenic (10). Although still controversial, emerging data also suggests that retinoid treatment may be linked to the development or exacerbation of irritable bowel syndrome (42) and depression and suicidal tendencies (45). Lastly, we have shown a detrimental effect on antioxidant defense as indicated by lower plasma glutathione concentrations at 24 wk. Hepatic levels may be more sensitive to retinoid treatment as de Oliviera et al (46) reported decreased hepatic glutathione concentrations after only 7 d of treatment, thereby creating an pro-oxidant environment.

Intermittent treatment has been studied as an attractive option for therapeutic use due to associated negative side effects. When intermittent oral treatment with isotretinoin was provided for 6 mo, it was equally efficacious in reducing moderate acne as daily treatment, but it was not as successful in ameliorating severe acne (47). Although daily oral isotretinoin treatment remains the gold standard of systemic acne treatment (48), the side effects associated with the intermittent treatment were less frequent and less severe than those associated with daily treatment (47). The data from our study suggests that while overt clinical signs of vitamin A toxicity may not be present, metabolic perturbations and lowered hematocrit develop after just a few weeks and persist even with non-daily dosing of RA. Antioxidant defense is also compromised after long-term supplementation. Although symptoms of retinoid toxicity generally subside with no ill effects after withdrawal of RA treatment (19), there remains the possibility that perturbations experienced during treatment could put the patient at greater risk for complications in the future.

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CHAPTER 4: Type I diabetes leads to tissue-specific DNA hypomethylation in male rats

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Abstract

Numerous perturbations of methyl group and homocysteine metabolism have been documented as an outcome of diabetes. It has also been observed that there is a transition from hypo- to hyperhomocysteinemia in diabetes, often concurrent with development of nephropathy. The objective of this study was to characterize the temporal changes in methyl group and homocysteine metabolism in the liver and kidney, as well as to determine the impact these alterations have on DNA methylation in type 1 diabetic rats. Male Sprague-Dawley rats were injected with streptozotocin (60 mg/kg body wt) to induce diabetes and samples were collected at 2, 4 and 8 wk. At 8 wk, hepatic and renal betaine-homocysteine *S*-methyltransferase activities were greater in diabetic rats, whereas methionine synthase activity was lower in diabetic rat liver and there was no difference in kidney. Cystathionine β-synthase abundance was greater in the liver, but less in the kidney of diabetic rats. Both hepatic and renal glycine *N*-methyltransferase (GNMT) activity and abundance were greater in diabetic rats; however, changes in renal activity and/or abundance were present only at 2 and 4 wk, whereas hepatic GNMT was induced at all timepoints. Most importantly, we have shown for the first time that genomic DNA was hypomethylated in the liver, but not the kidney in diabetic rats. These results suggest that diabetes-induced perturbations of methyl group and homocysteine metabolism lead to functional methyl deficiency, resulting in the hypomethylation of DNA in a tissue-specific fashion.

Key words: diabetes • DNA methylation • folate • homocysteine • rat

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Introduction

Perturbation of methyl group metabolism is associated with numerous pathologies, including cancer, cardiovascular disease, neurological problems, and birth defects (1-4). Methyl group supply is determined largely by two factors: dietary methyl intake and methyl group utilization. The major dietary methyl donors are methionine, choline, and betaine (5). Production of 5-methyltetrahydrofolate (5-CH_3-THF) from 5-methylenetetrahydrofolate via methylenetetrahydrofolate reductase (MTHFR) serves as an endogenous methyl donor. Methyl group metabolism consists of four processes: transmethylation, remethylation by folate/B_{12}-dependent or -independent means, and transsulfuration. Transmethylation reactions are essential for many biological processes and involve the transfer of a methyl group from *S*-adenosylmethionine (SAM) to various substrates including nucleic acids, lipids, and proteins by methyltransferases. All SAM-dependent methyltransferase reactions ultimately result in the generation of *S*-adenosylhomocysteine (SAH) and subsequently homocysteine, which can be remethylated back to methionine or irreversibly catabolized by transsulfuration. Remethylation of homocysteine to generate methionine occurs via folate-dependent and/or –independent pathways. For folate-dependent remethylation, the B_{12}-dependent enzyme methionine synthase (MS) utilizes a methyl group from 5-CH_3-THF. Betaine-homocysteine *S*-methyltransferase (BHMT) catalyzes the folate-independent remethylation of homocysteine using betaine, a methyl group donor derived from choline oxidation. Catabolism of homocysteine via the transsulfuration pathway begins with the irreversible conversion to cystathionine by cystathionine β-synthase (CBS).

Because methyl group metabolism is important in health and disease, identifying and understanding factors that have a regulatory role is essential. Recently, diabetes has emerged as a condition characterized by disrupted methyl group metabolism. In the acute diabetic state, the expression and activity of both hepatic phosphatidylethanolamine *N*-methyltransferase (PEMT) and glycine *N*-methyltransferase (GNMT) were elevated. Because PEMT and GNMT represent key SAM-dependent enzymes for phosphatidylcholine synthesis and regulation of methyl group metabolism respectively, this suggested that transmethylation was increased (6-9). Inappropriate upregulation of GNMT would be expected to lead to wastage of methyl groups due to incorporation of the methyl groups into sarcosine. For both type 1 and type 2 diabetic rat models, MS activity was decreased, whereas BHMT activity was markedly increased (7-10). Elevated expression of CBS as a

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function of diabetes also suggests enhanced catabolism of homocysteine via the transsulfuration pathway (7,9,11,12). The net effect of these alterations was that hepatic levels of both methionine and betaine were decreased (9,11), suggesting the potential development of methyl deficiency. Furthermore, both the activity of MTHFR in lymphocytes and the ratio of intracellular SAM:SAH, an indicator of transmethylation potential, in erythrocytes were decreased in diabetic nephropathy with an inverse relation to the severity of illness (13). Transmethylation flux has also been shown to be suppressed in diabetics with renal dysfunction (14). Taken together, this suggests that a diabetic condition has a profound impact on methyl group metabolism and that both the liver and kidney may be important in the pathophysiological progression of the disease.

To date, most studies have been conducted early in the progression of the disease or were cross-sectional. The goal of this study was to determine whether the perturbations of methyl group metabolism associated with a diabetic condition are sustained over time and how this might contribute to a functional methyl group deficiency, ultimately resulting in genomic hypomethylation and altered expression of proteins associated with DNA methylation, such as DNA methyltransferase 1 (DNMT1) (15). Based on evidence that aberrations of methyl group metabolism were more severe in diabetics with renal dysfunction (13,14), it was also of interest to determine whether these effects were tissue-specific.

Materials and Methods

Chemicals and reagents. Reagents were obtained as follows: [^{14}C-*methyl*]-betaine was obtained from Moraveck; Coomassie Plus Protein Reagent, Pierce Chemical; DL-homocysteine thiolactone, Sigma-Aldrich Chemical; DNMT1 (K-18) antibody and goat anti-rabbit IgG-horseradish peroxidase, Santa Cruz Technologies; enhanced chemiluminescence Western blotting detection reagents and 5-[^{14}C]-methyl-THF, Amersham Pharmacia; goat anti-mouse horseradish peroxidase secondary antibody Southern Biotechnology; phenylmethylsulfonyl fluoride (PMSF), Calbiochem; and *S*-adenosyl-L-[^{3}H]-methionine, New England Nuclear. The GNMT antibody was provided by Y-M.A. Chen (National Yang-Ming University, Taipei, Taiwan) (16). The CBS antibody was provided by J.

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Kraus (University of Colorado Health Sciences Center). All other reagents were of analytical grade.

Rats and diets. All animal experiments were approved by and conducted in accordance with guidelines established by Iowa State University Laboratory Animal Resources. Thirty male Sprague-Dawley rats (100-124 g) were housed individually in plastic cages with a 12-h light-dark cycle. All rats were given *ad libitum* access to a semi-purified diet (17) and water. After an acclimation period (4 d), rats were randomly assigned to either the control or diabetic treatment group for a total treatment time of 2, 4, or 8 wk (5 rats per group per timepoint). One diabetic rat assigned to wk 8 died during wk 6. All rats were given a single intraperitoneal injection of either vehicle (10 mmol/L citrate buffer, pH 4.5) or streptozotocin (STZ, 60 mg/kg body wt), for induction of type 1 diabetes. At each timepoint, rats were anesthetized with an intraperitoneal injection of ketamine and xylazine (90 and 10 mg/kg body wt respectively), and heparinized whole blood samples were collected via cardiac puncture. An aliquot of whole blood was saved for analysis of blood glucose (# 510, Sigma-Aldrich). The remaining blood was centrifuged at 4,000 × *g* for 5 minutes, followed by removal of the plasma layer and storage at -20℃ unti l analysis. The liver and left kidney were rapidly excised and a portion of each tissue was homogenized in ice-cold buffer containing 10 mmol/L sodium phosphate (pH 7.0), 0.25 mol/L sucrose, 1 mmol/L EDTA, 1 mmol/L sodium azide and 0.1 mmol/L PMSF. After centrifugation at 20,000 × *g* for 30 min at 4℃, β-mercaptoethanol was added to the supernatants to a final concentration of 10 mmol/L. Remaining tissues were snap frozen in liquid nitrogen and stored at -70℃ prior to isolation of genomic DNA (cat # A1125, Promega).

GNMT and PEMT GNMT and PEMT represent two important SAM-dependent transmethylation enzymes that also function to regulate homocysteine and methyl group metabolism. GNMT activity was determined based on the method of Cook and Wagner (18) with minor modifications (17). GNMT abundance was assessed using immunoblotting and subsequent chemiluminescent detection. The 32 kDa subunit was separated using SDS-PAGE. The protein was transferred to a nitrocellulose membrane and incubated with GNMT antibody followed by incubation with goat anti-mouse horseradish peroxidase secondary

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antibody. Densitometric analysis (SigmaGel Software, SPSS, Chicago, IL) was used for relative quantification of GNMT abundance. For PEMT activity, frozen liver was homogenized in 10 mmol/L Tris-HCl (pH 7.4) and 0.25 mol/L sucrose. Following centrifugation at 16,000 × *g* for 20 min at 4℃, the supernatant was removed and centrifuged at 100,000 × *g* for 60 min at 4℃. The resulting microsomal pellet w as resuspended in 0.25 mol/L sucrose. The enzymatic activity of PEMT was determined using the method of Duce *et al* (19) with minor modifications (6).

BHMT and MS Remethylation of homocysteine to methionine by the folate/B12-independent and dependent pathways is controlled by the enzymes BHMT and MS, respectively. For both enzyme assays, fresh homocysteine solutions (100 mmol/L) were prepared daily by dissolving a thiolactone derivative in 2 mol/L sodium hydroxide followed by neutralization with saturated monopotatssium phosphate (20). As described previously (7), BHMT and MS activities were assessed by radioisotopic assays utilizing [*methyl*-^{14}C-] betaine (20) and [*methyl*-^{14}C-] tetrahydrofolate (21), respectively.

CBS The irreversible catabolism of homocysteine by the transsulfuration pathway is initiated by the reaction catalyzed by CBS. Abundance of CBS was determined using immunoblotting and chemiluminescence in a method similar to that described for GNMT (22). The 63 kDa subunit of CBS was isolated using SDS-PAGE. After electrophoretic transfer to nitrocellulose, the immunoblot was incubated with a polyclonal CBS antibody followed by incubation with goat anti-rabbit horseradish peroxidase secondary antibody and subsequent chemiluminescent and densitometric analysis.

Homocysteine, SAM, and SAH analysis Derivatization of plasma samples was performed for determination of plasma homocysteine as described by Ubbink et al (23) with minor modifications (7). Homocysteine was analyzed by HPLC with fluorometric detection by injecting 100 µL of sample onto a µBondapak C_{18} Radial-Pak column (Waters, Milford, MA) using a mobile phase of 40 mL/L acetonitrile in 0.1 mol/L potassium phosphate buffer (pH 2.1). The addition of *N*-acetylcysteine (1 mmol/L) to each sample prior to derivatization

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served as an internal standard. For analysis of hepatic SAM and SAH concentrations, liver samples were prepared using the method of Fell et al (24) and SAM and SAH were separated and quantified by reverse-phase HPLC with UV detection as described (7).

DNA methylation status Digestion of hepatic and renal DNA followed by cytosine extension was performed as described (25) for assessment of DNA methylation status. DNA (1.0 µg) was digested using the methylation-sensitive restriction enzymes *Hpa*II and *BssH*II (New England Biolabs) for determination of global and CpG island methylation, respectively. For the cytosine extension assay, a reaction mixture of the DNA digest, 10X PCR Buffer II (without $MgCl_2$), 25 mmol/L $MgCl_2$, 0.5 U Amplitaq DNA Polymerase (Applied Biosystems), and [^{3}H]-dCTP was incubated at 55˚C for 1 h. Following incubation, samples were applied to Whatman DE-81 ion exchange filter paper and washed in 0.5 mol/L sodium phosphate buffer (pH 7.0) three times, dried, and ^{3}H incorporation was assessed using liquid scintillation counting.

DNMT1 abundance The Western blotting procedure described for GNMT and CBS was also used to determine the DNMT1 abundance, with the following modifications. For DNMT1, the primary and secondary antibodies used were goat polyclonal anti-DNMT and donkey anti-goat, respectively.

Statistical analysis For each timepoint, the mean values of each treatment group were subjected to a Student's *t* test (SigmaStat, SPSS, Chicago, IL). A Mann-Whitney rank sum test was used when variances were unequal. Correlations were determined using the Pearson product moment correlation method. Differences were considered significant at $P < 0.05$.

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Results

Methyl group metabolism is perturbed by diabetes

Blood glucose levels were greater in diabetic rats than control rats at all timepoints (72, 151, and 185% greater than control at wk 2, 4, and 8, respectively, **Table 1**). Plasma homocysteine levels were lower in the diabetic group at all timepoints. (76, 65, and 53% of control at wk 2, 4, and 8, respectively, **Table 1**).

Table 1. Circulating concentrations of glucose and homocysteine in control and diabetic rats.[1]

	Time,wk		
	2	**4**	**8**
Blood glucose		*mmol/L*	
Control	12.2 ± 0.3	9.7 ± 0.5	9.8 ± 0.7
Diabetic	21.0 ± 1.7*	24.4 ± 2.8*	27.9 ± 1.1*
Plasma homocysteine		*µmol/L*	
Control	9.1 ± 0.3	10.0 ± 2.0	7.4 ± 0.8
Diabetic	2.2 ± 0.2*	3.5 ± 0.5*	3.5 ± 0.6*

[1] Values are mean ± SEM (n= 4-5). *Different from control at a given timepoint, $P < 0.05$.

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Consistent with earlier short-term studies (7,8,10), remethylation, by both folate-dependent and –independent pathways, was also altered by a chronic diabetic state. At 8 wk, hepatic BHMT activity was elevated (95%) and hepatic activity of MS was markedly lower (81%) in diabetic rats compared to controls (**Table 2**). In the kidney, BHMT activity was greater in diabetic rats than controls, but there was no significant change in MS activity. It should be noted that renal BHMT activity level in both control and diabetic rats was very low and thus the metabolic consequences of this increase may be minimal.

There was a sustained increase in hepatic CBS abundance in the diabetic rat, with an increase of 35% at 8 wk (**Fig. 1**). In contrast, renal CBS abundance was decreased 33% at 8 wk in the diabetic group compared to controls, which would also be expected to

Table 2. Hepatic and renal remethylation enzyme activity levels in control and diabetic rats at 8 wk after induction of diabetes.[1]

	Control	**Diabetic**	**P-value**
BHMT Activity		*nmol Met/(h • mg protein)*	
Hepatic	73 ± 10	142 ± 16	0.006
Renal	0.3 ± 0.1	1.1 ± 0.1	<0.001
MS Activity		*pmol/(min • mg protein)*	
Hepatic	24.0 ± 2.9	4.5 ± 1.6	<0.001
Renal	209 ± 28	148 ± 24	0.149

[1] Values are mean ± SEM, (n = 4-5).

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contribute to a sparing of methyl groups in the kidney by reducing the catabolism of homocysteine. For both tissues, similar changes were also found in diabetic rats at 2 and 4 wk (data not shown), consistent with previous short term studies (7,11,12).

The activity and/or abundance of the SAM-dependent methyltransferases (i.e. PEMT, GNMT) assessed were elevated as a result of a diabetic condition. Hepatic PEMT activity was elevated 31% at 8 wk (diabetic vs. control, 232 ± 9 vs. 177 ± 13 pmol/(min • mg protein), $P = 0.012$), which is similar to results from an acute (1 wk) study in diabetic rats (6).

The activity and abundance of hepatic GNMT were greater in the liver of the diabetic rat compared with control values at all timepoints (**Fig. 2A**), although the magnitude was diminished with time. The temporal pattern of GNMT induction by diabetes was tissue-

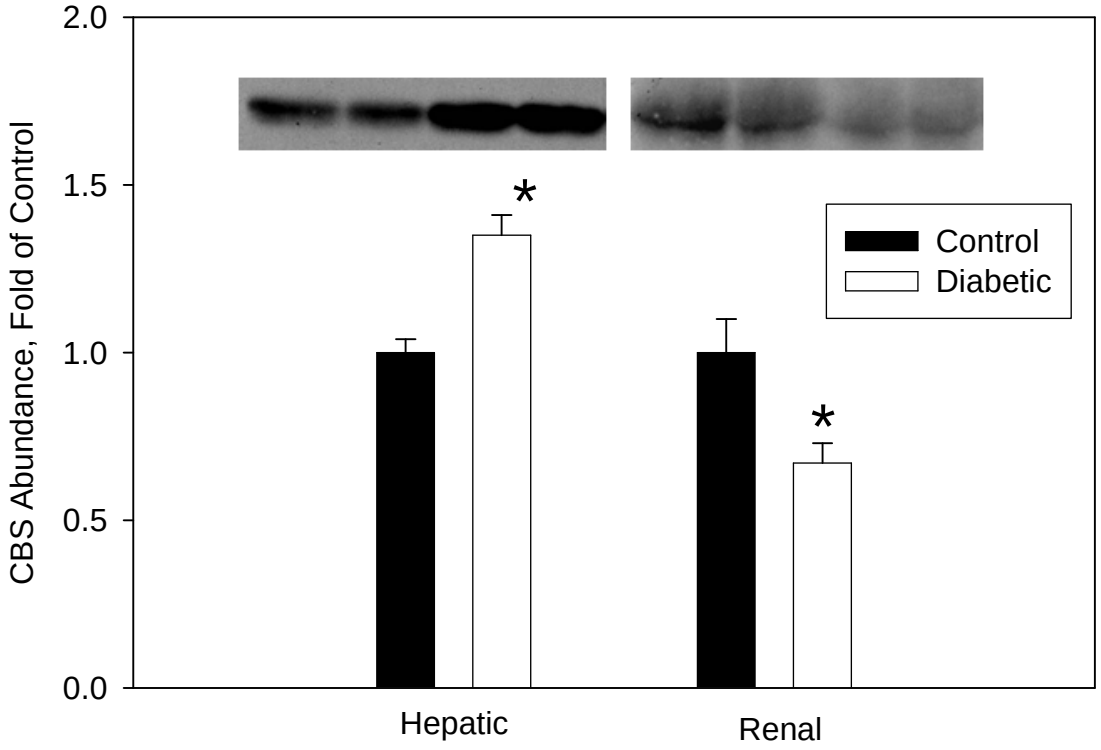

Figure 1. Hepatic and renal cystathionine β-synthase (CBS) abundance in control and diabetic rats at 8 wk after induction of diabetes. Values are mean ± SEM, (n= 4-5). *Different from control, $P < 0.05$. A representative blot is shown.

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specific. Renal GNMT activity was elevated at 2 and 4 wk in diabetic rats, but was not different from control values at 8 wk (**Fig. 2B**). Likewise, renal GNMT abundance was only greater at the 2 wk timepoint in diabetic rats.

DNA methylation status is affected by a diabetic condition in the liver, but not the kidney

DNMT1 protein abundance and SAM and SAH concentrations in the liver, as well as hepatic and renal global and CpG island methylation status were assessed as indicators of methyl group balance. There was no difference in hepatic SAH concentrations, but both hepatic SAM levels and the SAM:SAH ratios were slightly lower in diabetic rat liver compared to the control; however, this was not statistically significant (**Table 3**). However, hepatic DNMT1 abundance was 45% greater in diabetic rats than in controls at 8 wk (**Table 3**).

DNA methylation status was assessed using the cytosine extension DNA methylation assay (25), wherein endogenous DNA hypomethylation is indicated by an increase in [^{3}H]-dCTP incorporation. For liver, there was no significant difference in genomic DNA methylation between control and diabetic rats at 2 wk. However, there was a trend toward greater [^{3}H]-dCTP incorporation in diabetic rats at 4 wk ($P = 0.074$), and a significant increase of 70% in diabetic rats compared to controls at 8 wk (**Fig. 3**, $P = 0.004$). Hepatic CpG island DNA was hypomethylated in diabetic rats at 2 wk; however, there were no differences in CpG island methylation status at 4 or 8 wk (**Appendix A**). Interestingly, there was a strong positive correlation between the induction of hepatic GNMT activity and the degree of CpG methylation in the liver for all timepoints combined ($n = 29$, $r = 0.74$, $P < 0.01$). In marked contrast to hepatic tissue, there were no differences in renal DNA methylation status at any timepoint. Taken together, this data suggests that perturbations of hepatic methyl group metabolism by a diabetic condition were sustained through 8 wk, with subsequent alterations of DNA methylation status and elevated DNMT1 abundance, whereas the kidney appears to be less sensitive.

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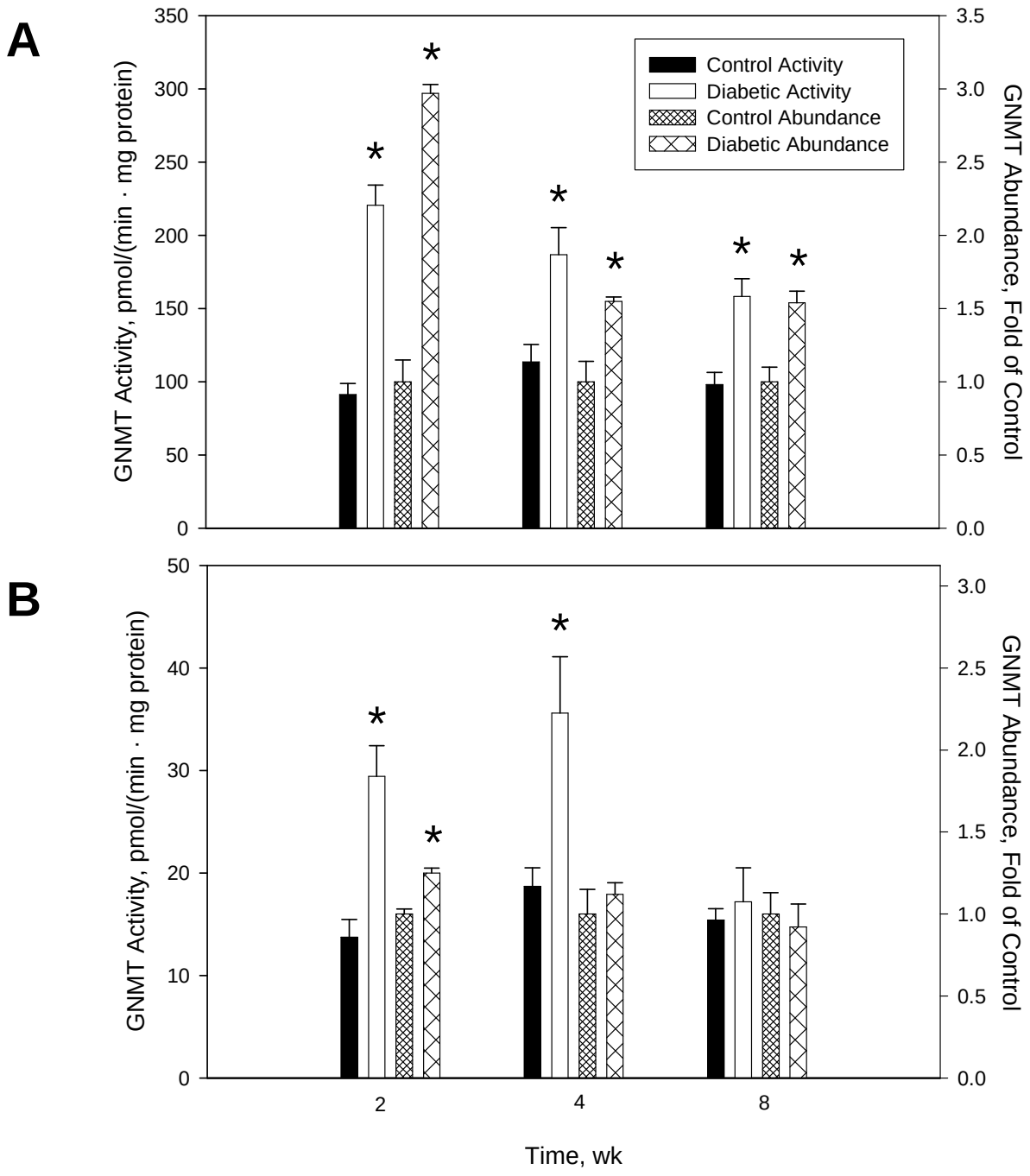

Figure 2. Hepatic GNMT activity and abundance in the liver (A) and kidney (B) of control and diabetic rats at 2, 4, and 8 wk after induction of diabetes. Values are mean ± SEM (n= 4-5). *Different from control at a given timepoint, $P < 0.05$.

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Table 3. SAM and SAH concentrations and DNMT1 abundance in the liver of type 1 diabetic male rats at 8 wk.

	Control	Diabetic
SAM *nmol/g liver*	28.0 ± 4.5	22.3 ± 5.8
SAH *nmol/g liver*	4.8 ± 0.5	5.5 ± 0.8
SAM:SAH	6.4 ± 1.4	4.6 ± 1.4
DNMT1 abundance *Fold of control*	1.00 ± 0.16	1.46 ± 0.05*

[1] Values are mean ± SEM, (n = 4-5). *Different from control, $P < 0.05$.

Discussion

Diabetes and its progression have been shown to be associated with secondary pathologies, including both micro- and macrovascular complications (26). Based on our previous acute diabetes studies that indicated aberrant methyl group metabolism in the liver (6-8), we postulated here that a chronic diabetic condition would ultimately result in more overt methyl group deficiency. Ultimately, this would be expected to compromise important SAM-dependent transmethylation reactions, such as the methylation of DNA. Indeed, global DNA hypomethylation was observed in the rat liver 8 wk after the induction of diabetes. To our knowledge, this is the first report demonstrating an association between diabetes and genome-wide epigenetic alterations of DNA. This finding may have significant implications for mechanistically linking diabetes to complications that are known to be influenced by DNA

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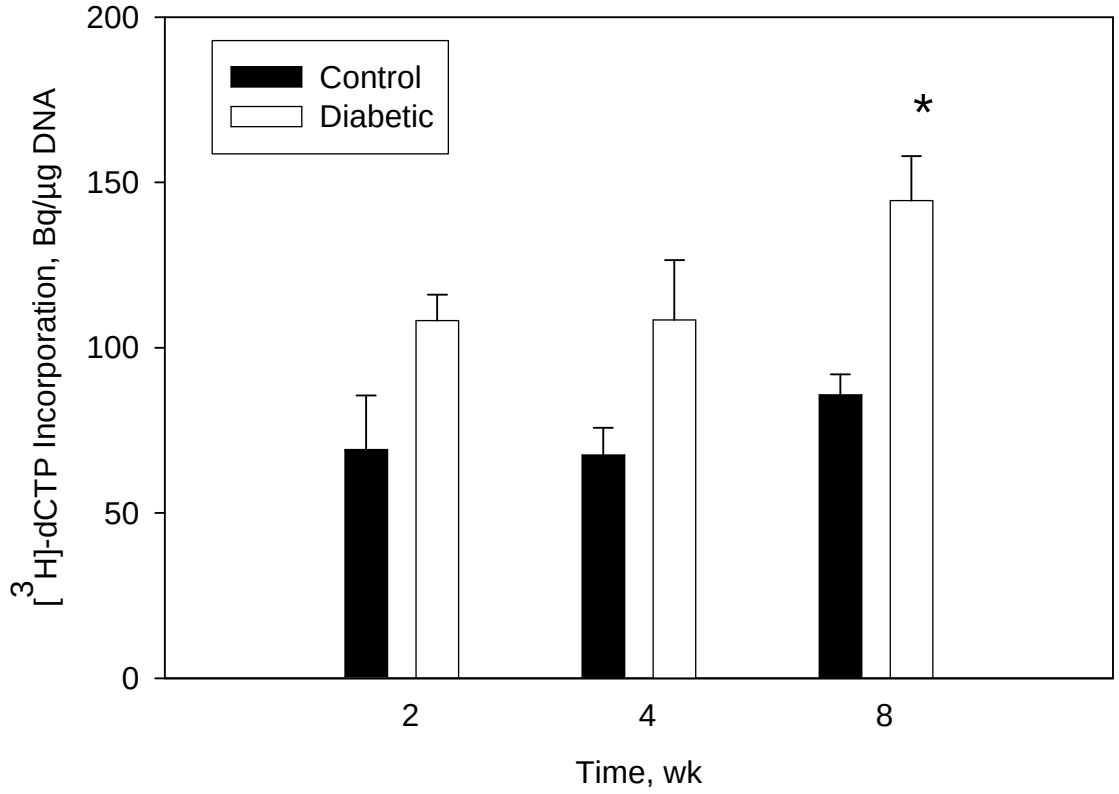

Figure 3. Genomic DNA methylation in the liver of control and diabetic rats at 2, 4, and 8 wk after induction of diabetes. Greater incorporation of [^{3}H]-dCTP indicates a greater degree of endogenous hypomethylation. Values are mean ± SEM (n= 4-5). *Different from control at a given timepoint, $P < 0.05$.

methylation and aberrant gene expression, such as cardiovascular disease and cancer (27-29).

In this study, plasma homocysteine concentrations in diabetic rats were lower than those in the controls throughout the treatment period. This finding is consistent with an earlier report which has shown that 10 wk after induction of diabetes, plasma homocysteine levels remained lower in diabetic rats than controls despite the onset of early renal dysfunction as evidenced by elevated urinary protein (30). Here, using plasma creatinine as an estimate of the glomerular filtration rate (GFR), diabetic hyperfiltration was present as

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evidenced by lower plasma creatinine levels (**Appendix A**). Typically, plasma homocysteine levels are inversely related to the GFR; thus, during the hyperfiltration that occurs in early diabetes in both humans and animal models, hypohomocysteinemia is observed (31-33). However, there was not a significant correlation between plasma homocysteine and creatinine concentrations ($r = 0.279$, $P = 0.15$), suggesting that greater excretion of homocysteine due to hyperfiltration is not the only determinant of circulating homocysteine concentrations. This conclusion was also supported by the multivariate analysis of Wollesen et al (33).

Given the role of the kidney in homocysteine balance (13,14,31), it was of interest to determine the effects of a diabetic condition in both the liver and the kidney. Hepatic perturbations of methyl group metabolism were sustained throughout the duration of the study, whereas the alterations in renal methyl group metabolism were more transient and no change in DNA methylation was observed. In support of this finding, genomic hypomethylation has been observed in the liver, but not the kidney, of rats fed a methyl-deficient diet (34). Dietary-induced methyl deficiency is characterized by: genomic and regional hypomethylation; perturbed expression of DNA methyltransferases and methyl-binding proteins; aberrant histone modifications; uracil misincorporation; and DNA strand breaks (15,34-37). All of these alterations contribute to genomic instability and thus play an important role in carcinogenesis. Induction of DNMT1 is characteristic of dietary-induced methyl deficiency (15,37) and was also observed in our diabetic rats at wk 8 in this experiment.

The results presented here suggest that alterations of methyl group metabolism were sustained in the liver, whereas the kidney was less sensitive to such changes. Despite differences in the pathology of type 1 vs. type 2 diabetes, the characteristic changes in methyl group metabolism appear to be similar for both conditions. The increases in hepatic GNMT, PEMT, BHMT, and CBS activity and/or abundance, as well as the decrease in hepatic MS activity has been previously described in acute models of type 1 and/or 2 diabetes (6-12). The data presented here suggests that these hepatic effects are sustained for at least 8 wk. However, in the kidney there was only transient induction of GNMT, a decrease in CBS abundance, and no difference in either MS activity or DNA methylation status. Taken together, the diabetic rat model shows similarities to methyl-deficient rat model, such that hepatic methyl group and methionine metabolism is perturbed for at least 8

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wk, resulting in genomic hypomethylation and aberrant expression of DNMT1. Moreover, these effects are clearly tissue-specific.

The changes induced by a diabetic condition could have secondary consequences, particularly based on the collective changes in GNMT and DNA methylation. GNMT is multifunctional and in diabetes it may be upregulated to generate pyruvate from methionine for gluconeogenesis, especially as its expression is limited to gluconeogenic tissues (38); it is also a proposed regulator of the SAM:SAH ratio (39). Thus, upregulation of GNMT in diabetes would be expected to decrease the SAM:SAH ratio, thus limiting the intracellular transmethylation potential and numerous transmethylation reactions, including DNA methylation. Although the hepatic SAM:SAH in diabetic rats in this study was not significantly different, DNA hypomethylation was clearly evident. For liver, dietary methyl deficiency is an independent carcinogen and has long been known to cause hypomethylation in the promoter regions of oncogenes (40,41). With the functional methyl deficiency associated with a diabetic condition, it would be expected that diabetes may also be associated with increased risk of hepatocellular carcinoma. This is supported by several epidemiological studies examining the relationship between diabetes and cancer have found an increased incidence of liver cancers of both type 1 and/or type 2 diabetic patients (42-45). In addition to hepatic carcinogenesis, hypomethylation of DNA and alterations of methyl group metabolism have also been implicated in the development of vascular diseases. Global DNA hypomethylation has been observed in advanced atherosclerosis in the rabbit and mouse, as well as in humans (27,28). Aberrant DNA methylation patterns have been detected early in the development of the disease (46) and become more prevalent with the progression of atherosclerosis, thus suggesting that epigenetic mechanisms may play a critical role in the atherosclerotic pathogenesis. It remains to be determined if diabetes-induced changes in DNA methylation are linked to cardiovascular disease.

Regulation of specific enzymes of methyl group metabolism may also impact DNA methylation status and pathogenesis of disease. Deficiency of MS or MTHFR were both reported to have negative effects on cerebral vascular function and lipid deposition was found in the aorta of mice hetero- or homozygous for the MTHFR knockout (47,48). Both MTHFR mutants also had lower SAM:SAH ratios and regions of both DNA hypomethylation in several tissues, suggesting impaired methylation capacity (48). GNMT is proposed to be

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the primary regulator of methyl group supply and utilization and thus, aberrant regulation of GNMT activity might be expected to perturb DNA methylation status. A recent report by Martinez-Chantar et al (49) demonstrates that in GNMT-knockout mice methylation status of specific genes was increased. We found a similar relationship in the diabetic rat liver, such that lower levels of GNMT activity were correlated with a greater degree of CpG island methylation and upregulation of GNMT was associated with hypomethylation. It would appear that decreasing GNMT activity was associated with the silencing of genes, whereas increasing GNMT activity would favor the activation of genes.

DNA methylation has also been closely linked to patterns of histone methylation and other histone modifications which could also contribute to aberrant gene expression and development of disease (29). Recently it was reported that in lymphocytes cultured under high glucose, methylation patterns of histones were altered in the regions of several genes which may be associated with diabetes via signal transduction, transporter, inflammation, and oxidant stress pathways (50). Mechanistically, evidence suggests that aberrant expression of methyl-binding proteins, histone methyltransferases and histone acetylases are also involved (15,37,50).

In summary, we have shown for the first time that chronic alterations of methyl group metabolism concomitant with genomic hypomethylation in the rat liver as a result of type 1 diabetes. In contrast, the kidney was more resistant to perturbations of methyl group metabolism and no changes were found in renal DNA methylation. The identification of widespread genomic DNA hypomethylation is a particularly novel finding and supports our hypothesis that a functional methyl deficiency develops in a diabetic state and may have implications concerning gene expression, DNA stability, and the development of secondary complications, such as vascular diseases and tissue-specific carcinogenesis. Because regulation of GNMT appears to be a major determinant of DNA hypomethylation, it is of interest to note that the tissues shown to be susceptible to cancer development in diabetes (42-45) are the same tissues that are known to express GNMT (38). Future research efforts will be geared towards further characterizing these metabolic and epigenetic alterations to gain a better understanding of the consequences of these changes and identifying timely dietary interventions that might be successful in ameliorating negative effects.

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CHAPTER 5: Tissue-specific alterations of methyl group metabolism and DNA hypermethylation in the Zucker (type 2) diabetic fatty (ZDF) rat

A manuscript to be submitted to the American Journal of Physiology – Endocrinology and Metabolism

Kelly T. Williams and Kevin L. Schalinske

Abstract

Altered methyl group and homocysteine metabolism was tissue-specific, non-transient, and preceded hepatic DNA hypomethylation in streptozotocin (STZ)-induced type 1 diabetic rats. Similar perturbations of hepatic methyl group and homocysteine metabolism have been shown in the Zucker (type 2) diabetic fatty (ZDF) rat in the pre- and early diabetic stages, but the tissue specificity and effect on epigenetic regulation are unknown, particularly with respect to disease progression. With this objective in mind, ZDF and lean rats (ZDF/Gmi *fa/fa* and +/?) were euthanized at 12 and 21 wk of age, representing early and advanced diabetic conditions. At 12 wk, hepatic glycine *N*-methyltransferase (GNMT), methionine synthase, and cystathionine β-synthase (CBS) activity and/or abundance were increased, whereas plasma homocysteine was decreased in ZDF rats. At 21 wk, GNMT activity was increased in liver and kidney. In liver only, there was a trend toward increased CBS protein abundance (78%) and betaine-homocysteine *S*-methyltransferase mRNA expression (~100%). Phosphatidylethanolamine *N*-methyltransferase activity tended to be lower in ZDF liver despite a significant increase in mRNA abundance. Homocysteine levels were decreased in plasma and kidney, but not in liver, at 12 and 21 wk. In contrast to the hypomethylation observed in the STZ-diabetic rat liver, hepatic genomic DNA was hypermethylated at 12 and 21 wk in ZDF rats, concurrent with a trend toward increased DNMT1 mRNA expression ($p = 0.08$) at 21 wk. In conclusion, the progression of type 2 diabetes in the ZDF rat was associated with tissue- and disease stage-specific aberrations of methyl group and homocysteine metabolism, with persistent hepatic global DNA hypermethylation.

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Introduction

It is estimated that over 12% of the U.S. population is afflicted with diabetes, with type 2 diabetes representing 95% of cases (1). Diabetes has been identified as a condition that can profoundly alter methyl group and homocysteine metabolism (2,3). There are three primary pathways involved in homocysteine metabolism: transmethylation, remethylation via folate/B_{12}-dependent or –independent means, and transsulfuration (**Figure 1**). For transmethylation reactions, methionine is activated to *S*-adenosylmethionine (SAM), which serves as the universal methyl group donor for numerous methyltransferases, resulting in the methylation of substrates such as nucleic acids, lipids, and proteins. Methyltransferase reactions result in the generation of homocysteine, which can be remethylated back to methionine using methyl groups donated by either 5-methyltetrahydrofolate (5-CH_3-THF) or betaine. For folate-dependent remethylation, the B_{12}-dependent enzyme methionine synthase (MS) utilizes a methyl group from 5-CH_3-THF. Betaine-homocysteine *S*-methyltransferase (BHMT) catalyzes the folate-independent remethylation of homocysteine using betaine, a methyl group donor derived from choline oxidation. Alternatively, homocysteine can be catabolized through the transsulfuration pathway to cysteine, beginning with the irreversible conversion to cystathionine by cystathionine β-synthase (CBS).

We, and others, have previously shown that an early diabetic condition alters methyl group and homocysteine metabolism. Diabetes induced hepatic protein abundance and activity of glycine *N*-methyltransferase (GNMT) and phosphatidylethanolamine *N*-methyltransferase (PEMT, 4-9). Both enzymes have important roles in regulation of methyl groups and homocysteine: GNMT is proposed to be a regulator of the transmethylation potential via maintenance of the SAM:*S*-adenosylhomocysteine (SAH) ratio, PEMT is purported to be the greatest consumer of SAM-derived methyl groups (10) and catalyzes the sequential trimethylation of phosphatidylethanolamine to phosphatidylcholine. Induction of BHMT and CBS are also consistently observed in diabetic or glucocorticoid-treated conditions (4,5,7-9,11,12), whereas the effect of diabetes on MS is not consistent. In sum, these metabolic alterations result in hypohomocysteinemia in the early diabetic condition, likely owing in part to greater upregulation of transsulfuration and remethylation via BHMT such that it overwhelms increases in transmethylation. Importantly, similar aberrations are

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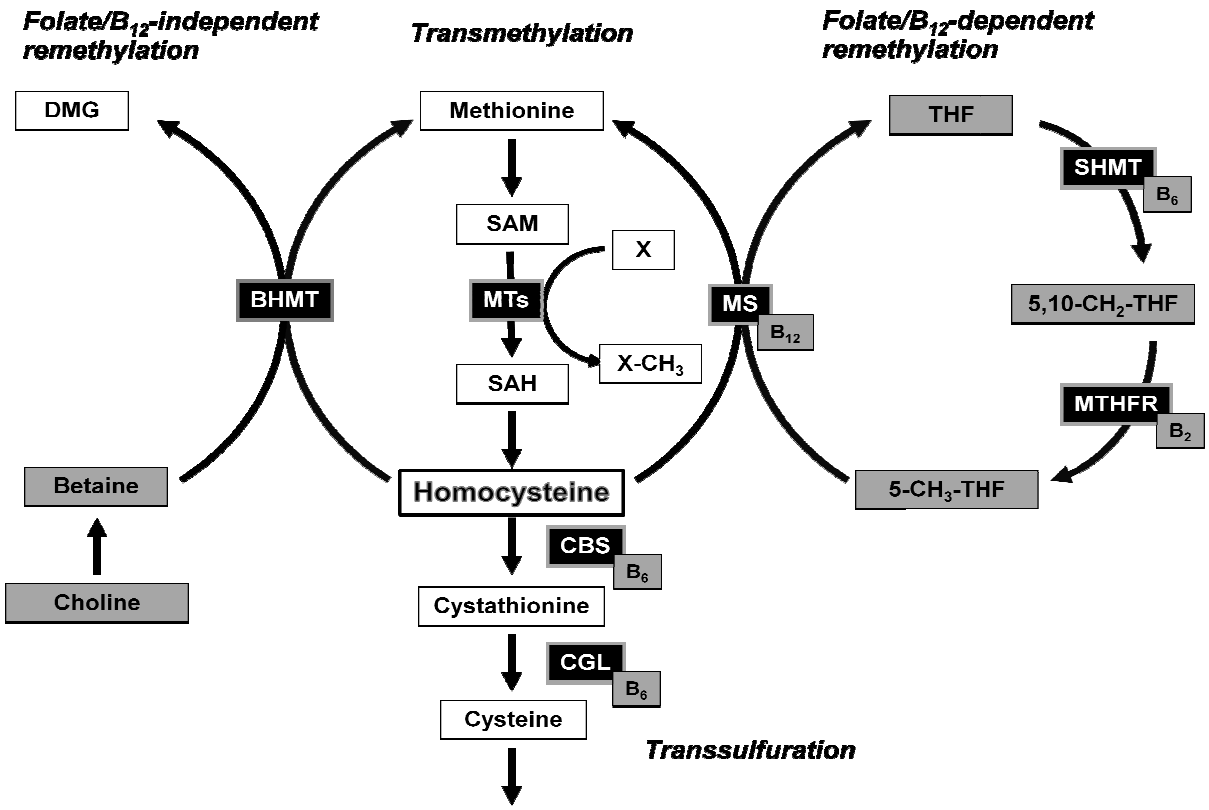

Figure 1. Methyl group and homocysteine metabolism. Enzymes shown in black boxes, B vitamin substrates and/or cofactors shown in gray boxes.

Abbreviations: 5-CH_3-THF, 5-methyltetrahydrofolate; 5,10-CH_2-THF, 5,10-methylenetetrahydrofolate; BHMT, betaine-homocystiene *S*-methyltransferase; CBS, cystationine β-synthase; CGL, cystathionine γ-lyase; DMG, dimethylglycine; MTHFR, methylenetetrathydrofolate reducttase; MTs, methyltrasnferases; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate; SHMT, serine hydroxymethyltransferase; X, methyl acceptor

observed in both type 1 and type 2 diabetic rat models (4,5,7-9), as well as type 1 and type 2 diabetic patients (13,14)

Progression of diabetes is associated with the development of secondary complications, including cardiovascular disease, nephropathy, neuropathy, and retinopathy. With the development of renal dysfunction in diabetic patients there is a shift from hypo- to hyperhomocysteinemia and suppression of MTHFR activity that increases with severity of nephropathy (14,15). Type 2 diabetics with nephropathy also have decreased flux through the transsulfuration pathway, which likely contributes to decreased homocysteine clearance (16). When studying the progression of diabetes in the streptozotocin (STZ)-induced type 1

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diabetic rat, we noted that the effect of diabetes was tissue-specific and sustained alterations of hepatic methyl group and homocysteine metabolism appeared to lead to a methyl deficient condition, as evidenced by genomic DNA hypomethylation in the liver (7).

The progression of type 2 diabetes from prediabetes to the early diabetic state in the Zucker (type 2) diabetic fatty (ZDF) rat has been shown nicely by Wijekoon et al. (9), however the advanced diabetic condition has yet to be fully characterized. Poirier et al (14) reported no differences between the type 1 and type 2 patients within their study, and the SAM:SAH ratio decreased with increasing severity of disease. Therefore, we hypothesized that ZDF rats with advanced diabetes would have perturbed methyl group and homocysteine metabolism, including chronic upregulation of GNMT, and develop a methyl-deficient condition. Furthermore, due to the functional methyl deficiency, the animals would have abnormal DNA methylation and expression of epigenetic regulatory proteins, similar to that observed in the STZ-diabetic rat (7) or rats fed methyl-deficient diets (16).

Materials and Methods

Chemicals and reagents Reagents were obtained as follows: 3T3 fully methylated DNA, *BssHII, HpaII,* and *MspI* restriction enzymes, New England Biolabs, Inc. (Ipswich, MA); 5-[^{3}H]-dCTP, MP Biomedicals (Solon, OH); enhanced chemiluminescence Western blotting detection reagents and 5-[^{14}C]-methyl-THF, Amersham/GE Healthcare (Piscataway, NJ); CBS antibody (H-300, sc-67154), goat anti-rabbit and goat anti-chicken horseradish peroxidase secondary antibodies, Santa Cruz Biotechnology, Inc (Santa Cruz, CA); and *S*-adenosyl-L-[^{3}H]-methionine, Perkin Elmer Life Sciences (Waltham, MA). All other reagents were of analytical grade.

Animals All animal protocols were approved by and conducted in accordance with the guidelines set forth by the Iowa State University Institutional Animal Care and Use Committee. The first set of experiments utilized rats which were purchased at 11 wk of age, allowed to acclimate for 1 wk, and terminated at 12 wk of age. For the second study, rats were purchased at 10 wk of age and sacrificed at 21 wk of age. Each study utilized six lean (ZDF/Gmi +/?) and six ZDF (ZDF/Gmi *fa/fa*) rats (Charles River Laboratories, Wilmington, MA) kept in individual cages with a 12:12 hr light:dark cycle and given *ad libitum* access to

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water and Purina 5008 diet. Animals were fasted for 12 hr prior to euthanasia. Rats were anesthetized by intraperitoneal injection with ketamine and xylazine (90 and 10 mg/kg respectively). Blood samples were then collected by cardiac puncture and tissues were collected including the liver, right kidney, and heart. Blood glucose levels were immediately assessed using a glucometer. Plasma and serum samples were collected by standard centrifugation methods and stored at -20℃. Serum insu lin levels were analyzed by Rat/Mouse Insulin ELISA Kit (Linco, Inc., St Charles, MO). A portion of the liver was immediately homogenized and cytosolic extracts prepared as previously described (7); the remainder of the liver and all other tissues were snap frozen in liquid nitrogen and subsequently stored at -70℃. Protein concentrations of extracts were determined using the Bradford assay with Coomassie Protein Plus Reagent (Thermo Scientific).

Enzyme assays Activities of GNMT, PEMT, and MS were determined using radioisotopic assays as previously described (5,6,7,17,18). Briefly, GNMT and MS activity in the liver, kidney, and heart were determined by incubation of cytosolic extracts with reaction mixes containing *S*-adenosyl-L-[^{3}H]-methionine and 5-[^{14}C]-methyl-THF, respectively. After halting the reaction, the unreacted, radiolabeled substrate was removed via sequestration by activated charcoal or anion exchange resin. Hepatic PEMT activity in microsomal extracts was assessed in a similar manner by determining the incorporation of *S*-adenosyl- L-[^{3}H]-methionine into the lipid fraction by the enzyme in microsomal extracts. Liquid scintillation counting was used to determine the extent of radiolabel incorporation for all assays.

Western blotting Relative protein abundance was determined using methods previously described (7) with minor modifications. All proteins were separated using SDS-PAGE. Ponceau S staining was used to verify equal loading. For assessment of GNMT, blots were incubated with a newly-procured affinity-purified chicken antibody (Aves Labs, Inc., Tigard, OR) directed against the following peptide sequence: KER WNR RKE PAF DK (GNMT residues #97-110), 1:40000 in TTBS/1% BSA. CBS primary antibody was prepared 1:400 in TTBS/1% BSA; goat anti-chicken or anti-rabbit horseradish peroxidase secondary antibodies were prepared at 1:5000 in TTBS. Relative protein amounts were quantified by densitometric analysis with QuantityOne software (Bio-Rad Laboratories, Hercules, CA).

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DNA methylation Using the method of Pogribny et al (19) and as described previously (7), digestion of DNA followed by cytosine extension was used for assessment of DNA methylation status in liver, kidney, and heart tissues. Global and CpG island DNA methylation were determined by digesting 1.0 µg DNA from the liver, kidney, or heart using the methylation-sensitive restriction enzymes *HpaII* and *BssHII*, respectively. For the cytosine extension assay, a reaction mixture of the DNA digest, 10X PCR Buffer II (without $MgCl_2$), 25 mmol/L $MgCl_2$, 0.5 U Amplitaq DNA Polymerase (Applied Biosystems), and [^{3}H]-dCTP was incubated at 55˚C for 1 h. Following incubation, samples were applied to Whatman DE-81 ion exchange filter paper and washed in 0.5 mol/L sodium phosphate buffer (pH 7.0) three times, dried, and ^{3}H incorporation was assessed using liquid scintillation counting. Samples were run in duplicate and reactions using either MspI-digested DNA or mouse 3T3 fully-methylated DNA with the appropriate restriction enzyme were used as controls. When compared to the *MspI* control, the degree of genomic methylation fell within the expected range of 70-90% (20).

Real-time RT-PCR Upon the first removal from storage, 0.1 g of frozen liver was taken and immediately preserved in 1 mL RNALater-ICE (Ambion, Inc, Austin, TX). This sample was subsequently used for RNA isolation using Trizol Reagent (Invitrogen, Carlsbad, CA). The reverse-transcription assay was performed using iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). The resulting cDNA was diluted 50x for use in the PCR reaction with iQ SYBR Green Supermix (Bio-Rad Laboratories). Primers used for RT-PCR (**Table 1**) were designed using PrimerQuest (Integrated DNA Technologies, Inc., Coralville, IA). Samples were run in duplicate, data were normalized to 18S control, and results were analyzed using the comparative C_t method.

Statistical analysis For each timepoint, the mean values of each treatment group were subjected to a Student's *t* test (SigmaStat, SPSS, Chicago, IL). A Mann-Whitney rank sum test was used when normality or equal variance test failed. Differences were considered significant at $P < 0.05$, trends were noted when $0.05 < P < 0.1$.

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Table 1. Real-time RT-PCR primers

Target	Primers (5' → 3')	
GNMT	F*:	ACA ACA AAG CCC ACA TGG TAA CCC
	R:	AGC CGA AAC TTA CTG AAG CCA GGA
PEMT	F:	TGT GCT CTC CAG CTT CTA TGC ACT
	R:	AGG GAA ATG TGG TCA CTC TGG ACT
MS	F:	TTG GCC TAC CGG ATG AAC AAA TGC
	R:	AGC CAC AAA CCT CTT GAC TCC TGT
BHMT	F:	ATC TGG GCA GAA GGT CAA TGA AGC
	R:	TGA CTC ACA CCT CCT GCA ACC AAT
CBS	F:	AAC ATG TTG TCC TCC CTG CTT GCT
	R:	TCG GCT TGA ACT GCT TGT AGA GGA
DNMT1	F:	TGT GGC AAG AAG AAA GGT GGC AAG
	R:	TGG ATG GAC TTG TGG GTG TTC TCA
DNMT3a	F:	AGA GTG TCT GGA ACA CGG CAG AAT
	R:	TGC TGG TCT TTG CCC TGC TTT ATG
DNMT3b	F:	TGC GCC TGC AAG ACT TCT TCA CTA
	R:	TGC AGG AAT CGC TGG GTA CAA CTT
MBD1	F:	CCT GCA CCT TTG TGC TGT GAG AAT
	R:	CAG TCT TTG CAC AAT GTC CTG CGT
MBD2	F:	TCA GAA GTA AAC CAC AGC TGG CGA
	R:	ACT AGG CAT CAT CTT GCC GCT TCT
MBD3	F:	GAA GAA GTT TCG CAG CAA GCC ACA
	R:	CAT CTT TCC CGT GCG GAA ATC GAA
MBD4	F:	AGC TAA ACC TCA GGA CAC GAA GCA
	R:	TTG GAC AGGCTG TTG CTA TCT GGA
MeCP2	F:	GCA GCA GCA TCA GAA GGT GTT CAA
	R:	TGC TTG GAA AGG CAT CTT GAC GAG
18S	F:	GAA CCA GAG CGA AAG CAT TTG CCA
	R:	ATG GTC GGA ACT ACG ACG GTA TCT

*F denotes forward primer, R denotes reverse primer.

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Results

General characteristics

The body weights of ZDF rats were significantly greater than their lean counterparts at 12 wk, whereas there was no significant difference in total body weight between groups at 21 wk (**Table 2**). For the 21 wk experimental group, it was noted that lean controls gained weight for the duration of the study, but the weight gain of ZDF rats plateaued or declined beginning around 16 wk of age (**Appendix B**), despite markedly increased food intake by ZDF rats observed at both 12 and 21 wks and throughout the experimental period. All ZDF rats were hyperglycemic, indicating that the animals were indeed diabetic at both 12 and 21 wk. There was a trend toward hyperinsulinemia in fasted ZDF rats at 12 wks of age, as would be expected in the early stages of type 2 diabetes with insulin resistance. The characteristics of the ZDF rat in the advanced diabetic stage were consistent with those reported by others (21).

Perturbed methyl group metabolism in the ZDF rat

Diabetes produced tissue- and time-specific changes in the expression and activity of GNMT and PEMT. GNMT activity and protein abundance were elevated in the liver of ZDF rats compared to lean controls at both 12 and 21 wk of age (**Figures 2 and 3**). GNMT mRNA abundance also tended towards an increase in the liver at 21 wks (**Table 3**). Renal GNMT activity was increased only at 21 wk ($p = 0.012$) without parallel increases in renal GNMT protein abundance, suggesting possible post-translational regulation of GNMT activity in the kidney. Unexpectedly, hepatic PEMT activity was unchanged in the diabetic rat at 12 wk and there was a trend towards decreased activity at 21 wk (lean vs. ZDF: 88 ± 10 vs. 62 ± 8 pmol/min x mg protein, $p = 0.071$). However, there was a trend towards increased hepatic PEMT mRNA abundance at 12 wk and a significant increase of 73% in PEMT mRNA abundance in ZDF rat liver at 21 wk (**Table 3**).

Removal of homocysteine via folate/B_{12}-dependent and independent remethylation or transsulfuration pathways was altered in the ZDF rat. BHMT mRNA abundance was increased in ZDF rat liver at 21 wk. CBS did not show any changes in mRNA abundance, but there was a tissue-specific effect on protein abundance (**Figure 3**). Similar to

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Table 2. Characteristics of ZDF (*fa/fa*) and lean (+/?) rats at 12 and 21 wks

	12 wk			21 wk		
	Lean	ZDF	p-value	Lean	ZDF	p-value
Body weight (g)	291 ± 7	332 ± 6*	0.001	396 ± 14	363 ± 6	0.06
Average food intake (g/d)	21.7 ± 0.3	40.8 ± 1.2*	0.002	20.0 ± 0.5	36.3 ± 0.7*	<0.001
Fasted blood glucose (mmol/L)	12.1 ± 0.9	26.2 ± 1.9*	<0.001	17.8 ± 1.1	30.2 ± 0.8*	<0.001
Fasted serum insulin (ng/mL)	0.32 ± 0.03	0.63 ± 0.10	0.054	0.44 ± 0.02	0.81 ± 0.13*	0.009

Data are means ± SEM (n = 3-6). * indicates a significant difference from control, $p < 0.05$.

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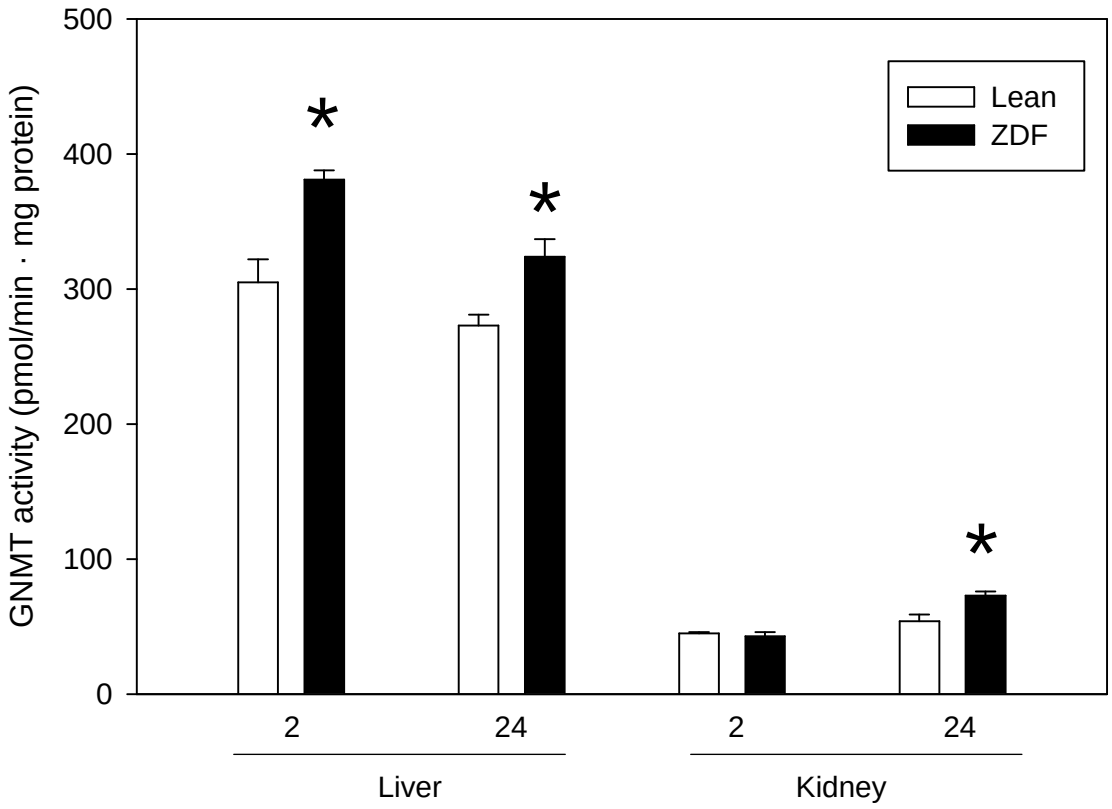

Figure 2. Increased hepatic and renal activity of GNMT in ZDF rat. Values are mean ± SEM (n= 6). *Different from control at a given timepoint, $P < 0.05$.

observations from the STZ-diabetic rat (7), CBS protein abundance was greater in the liver, with a trend towards decreased CBP abundance in the kidney when ZDF rats were compared to lean controls at 12 wk. At 21 wk, there were no significant differences in CBS protein levels between ZDF and lean rats in neither the early nor advanced diabetic conditions. In the early diabetic condition, there were slight trends towards increased MS activity and mRNA abundance in the liver (lean vs. ZDF, 416 ± 40 vs. 498 ± 21 pmol/(min • mg protein), p = 0.098, **Table 3**). No changes were observed in renal MS activity. With the exception of a trend towards increased MS activity in the heart at 21 wk (lean vs. ZDF, 286 ± 20 vs. 343 ± 22 pmol/(min • mg protein), p = 0.082), there were no significant changes in the heart. In both the early and advanced diabetic conditions, cardiac CBS and GNMT protein

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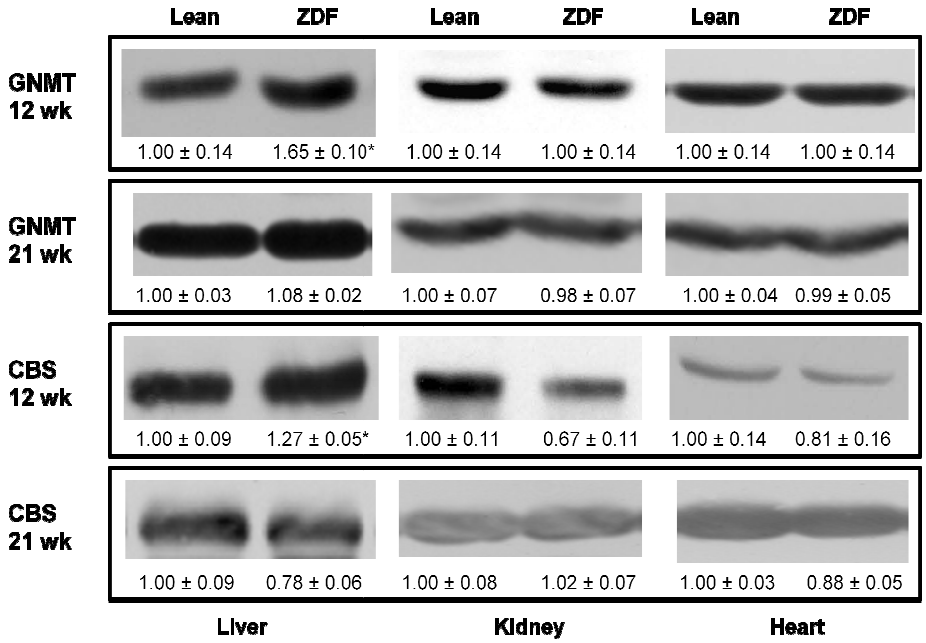

Figure 3. Tissue-specific alterations of GNMT and CBS protein abundance**.** Representative blots are shown. Values are mean ± SEM, (n=5-6). *Different from control, $P < 0.05$.

abundance were similar and no appreciable GNMT activity was detected.

Regulation of the enzymes of methyl group and homocysteine metabolism and concentrations of key intermediates of the pathways are interrelated. Accordingly, plasma and intracellular concentrations of homocysteine, glutathione, SAM, and SAH were also perturbed by a diabetic condition. There was an identical 37% increase in hepatic SAM concentrations and in the SAM:SAH ratio in ZDF rat liver relative to controls at 12 wk, although it must be noted that the increase in the SAM:SAH ratio did not achieve statistical significance (**Table 4**). Renal SAM concentrations were elevated in both the acute and advanced diabetic conditions. Additionally, SAH levels were lower in ZDF kidney at 12 wks. These perturbations likely both contributed to the increased SAM:SAH ratio in the kidney at both 12 and 21 wk.

Plasma total homocysteine concentrations were decreased 67 and 54% at 12 and 21 wk respectively (**Table 5**). There were no changes in hepatic concentrations of

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Table 3. Hepatic mRNA abundance of enzymes of methyl group and homocysteine metabolism in lean and ZDF rats at 12 and 21 wk of age.

	12 weeks, mean fold induction			21 weeks, mean fold induction		
Target	Lean	ZDF	p-value	Lean	ZDF	p-value
GNMT	1.00 ± 0.55	1.03 ± 0.23	0.477	1.00 ± 0.13	1.36 ± 0.16	0.054
PEMT	1.00 ± 0.40	3.31 ± 1.20	0.065	1.00 ± 0.18	1.73 ± 0.21*	0.012
MS	1.00 ± 0.15	1.91 ± 0.58	0.099	1.00 ± 0.27	1.30 ± 0.24	0.212
BHMT	1.00 ± 0.49	1.64 ± 0.47	0.186	1.00 ± 0.22	2.06 ± 0.54*	0.049
CBS	1.00 ± 0.54	0.77 ± 0.29	0.348	1.00 ± 0.28	1.10 ± 0.20	0.391

Data are means ± SEM, * indicates significant difference, $P < 0.05$

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Table 4. Elevations of hepatic and renal SAM concentrations in the ZDF rat relative to lean controls

	SAM (nmol/g tissue)			SAH (nmol/g tissue)			SAM:SAH Ratio		
	Lean	ZDF	p-value	Lean	ZDF	p-value	Lean	ZDF	p-value
Liver									
12 wk	51.6 ± 6.8	70.6 ± 5.2*	0.050	57.1 ± 2.6	58.5 ± 4.2	0.779	0.91 ± 0.13	1.25 ± 0.14	0.107
21 wk	62.5 ± 4.0	65.4 ± 7.4	0.744	63.5 ± 5.4	68.8 ± 7.2	0.563	1.03 ± 0.12	1.03 ± 0.17	0.998
Kidney									
12 wk	11.0 ± 0.9	19.2 ± 1.1*	<0.001	51.4 ± 6.2	39.6 ± 2.5*	0.026	0.22 ± 0.02	0.49 ± 0.04*	<0.001
21 wk	12.2 ± 0.9	18.7 ± 2.0	0.012	35.8 ± 1.9	32.8 ± 3.5	0.460	0.35 ± 0.03	0.59 ± 0.09	0.052

Data are means ± SEM, *indicates significantly different from the control via t-test or rank sum test, $P < 0.050$

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Table 5. Fasted plasma and tissue concentrations of total homocysteine and glutathione.

		Total homocysteine (µmol/L plasma or g tissue)			Total glutathione (µmol/L plasma or g tissue)		
	Time (wk)	Lean	ZDF	p-value	Lean	ZDF	p-value
Plasma	12	6.8 ± 0.3	2.2 ± 0.1*	<0.001	21.0 ± 1.2	19.6 ± 0.7	0.319
	21	3.6 ± 0.3	1.6 ± 0.1*	<0.001	13.6 ± 0.8	11.3 ± 1.0	0.115
Liver	12	155 ± 22	166 ± 10	0.664	5614 ± 261	7582 ± 413*	0.002
	21	168 ± 7	203 ± 20	0.240	7088 ± 263	7144 ± 235	0.876
Kidney	12	11.0 ± 4.1	4.1 ± 0.7*	0.030	41.8 ± 5.8	102.2 ± 4.1*	<0.001
	21	6.3 ± 0.9	3.2 ± 1.1	0.059	38.4 ± 10.3	65.4 ± 7.6	0.082

Data are means ± SEM, *indicates significantly different from the control via t-test or rank sum test.

homocysteine, whereas homocysteine concentrations were decreased 63% in the ZDF kidney at 12 wk. Most interestingly, there were significant increases in both hepatic and renal total glutathione concentrations at 12 wk. Renal glutathione levels were also elevated in ZDF rats at 21 wk, although this was only observed as a trend.

DNA hypermethylation and upregulation of DNMT1 in the diabetic rat liver

In contrast to the development of hepatic global DNA hypomethylation in the advanced STZ-diabetic rat (7), marked global DNA hypermethylation was characteristic of ZDF rat liver in both early and advanced diabetes (**Figure 4**). This effect was specific to genomic DNA in the liver; changes were observed in neither global DNA methylation levels in kidney and heart nor in CpG island methylation status as measured by the overall incorporation of [^{3}H]-dCTP. Likewise, the mRNA abundance of the DNMTs and MBDs were unaltered in early diabetes (**Appendix B**); however, DNMT1 mRNA abundance was increased 95% in the ZDF rat liver at 21 wk (**Table 6**). Despite minimal impact on the regulation of DNMTs and MBDs at the mRNA level, dysregulation of global DNA

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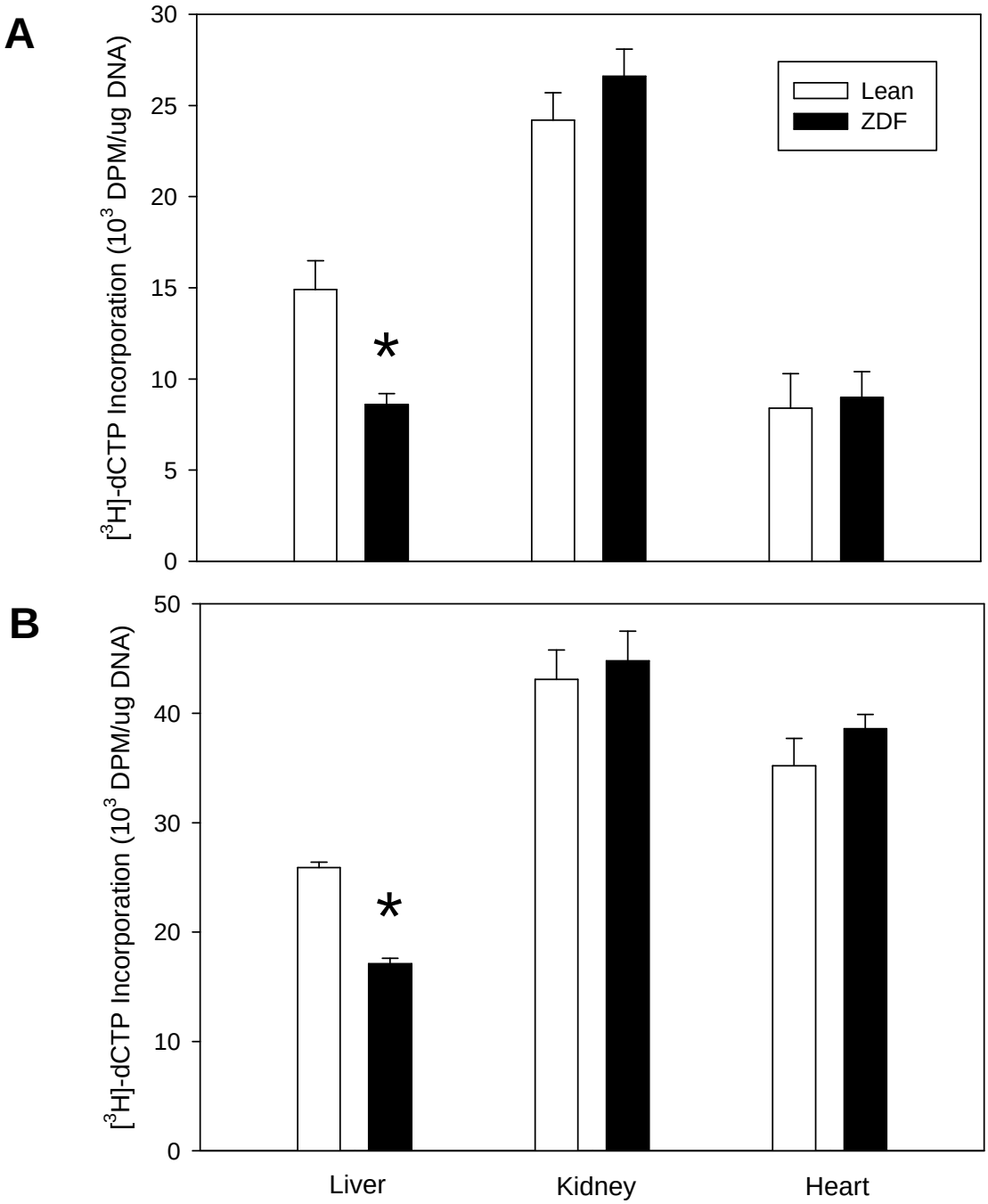

Figure 4. Hepatic hypermethylation of genomic DNA in ZDF rats at (A) 12 and (B) 21 wk of age**.** By the method of Pogribny et al (19), incorporation of [^{3}H]-dCTP is inversely related to the degree of methylation. Values are mean ± SEM (n=6). *Different from control, $P < 0.05$.

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Table 6. Hepatic mRNA abundance of epigenetic regulatory proteins in lean and ZDF rats at 21 wk of age.

	Mean fold induction		
Target	Lean	ZDF	p-value
DNMT1	1.00 ± 0.29	1.95 ± 0.37*	0.042
DNMT3a	1.00 ± 0.27	0.89 ± 0.12	0.353
DNMT3b	1.00 ± 0.34	0.92 ± 0.20	0.422
MBD1	1.00 ± 0.40	1.35 ± 0.29	0.241
MBD2	1.00 ± 0.17	1.02 ± 0.18	0.467
MBD3	1.00 ± 0.22	1.03 ± 0.14	0.458
MBD4	1.00 ± 0.58	0.19 ± 0.03	0.197
MeCP2	1.00 ± 0.47	0.72 ± 0.15	0.275

Data are means ± SEM (n=6), * indicates significant difference, $P < 0.05$

methylation patterns appear to be an early event in the pathogenesis of diabetes in the ZDF rat.

Discussion

Here we demonstrate that during the progression of type 2 diabetes in the ZDF rat, there are tissue-specific alterations of methyl group and homocysteine metabolism, with concurrent epigenetic dysregulation and abnormal concentrations of key intermediates. However, unlike the effects of the chronic type 1 diabetic condition in which genomic DNA hypomethylation develops in the liver, hepatic genomic DNA was hypermethylated in the ZDF rat, even in the early stages of the disease.

In early diabetes, both ZDF and type 1-diabetic rat models have been shown to exhibit similar perturbations hepatic methyl group and homocysteine metabolism (4-9) and which were also observed in the ZDF rats of this study at 12 wk. The net effect of these

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metabolic perturbations in the liver in early diabetes seems to favor the loss of methyl groups the catabolism of methionine to cysteine via induction of GNMT and the transsulfuration pathway. This might be the anticipated result based the abundance of evidence supporting GNMT as a regulator of the SAM:SAH ratio (22) and the known induction of CBS expression and activity by glucocorticoids and SAM (23,24). The upregulation of CBS may be a compensatory mechanism for self protection of the liver and kidney against the oxidative stress associated with a diabetic condition; CBS activity has been shown to be enhanced under conditions of oxidative stress (25). However, many studies have found depletion of glutathione in diabetic tissues (26), so the significance of this finding remains unclear.

It also might seem intuitive that when SAM levels have normalized - as occurred in the liver at 21 wk - that regulation would change to maintain transmethylation potential. As such, the induction of GNMT was not as great as it was at 12 wk, with only a 19% increase in activity and an 8% increase in protein abundance. There was also a significant increase in BHMT mRNA abundance that was not observed in the early diabetic condition. In diabetes, increases in BHMT mRNA abundance have previously been associated with increased BHMT activity (12), thereby we suggest remethylation by BHMT may be increased. Interestingly, PEMT was upregulated at the mRNA level, whereas PEMT activity was decreased. A decrease in production of homocysteine by PEMT and lesser induction of GNMT combined with an increase in BHMT activity might be expected to contribute to the hypohomocysteinemia that persisted in the advanced diabetic state despite no change of CBS protein abundance when ZDF rats were compared to controls.

The response of the kidney to a diabetic condition appears to be less stringently regulated. Whereas, enzymes of methyl group and homocysteine metabolism appeared to be regulated at a transcriptional and/or translational level with respect to SAM concentrations in the liver, there was a lesser impact on the enzymes of the kidney and furthermore, renal intracellular concentrations of SAM, SAH, homocysteine, and glutathione appeared to reflect those in the liver and the plasma. Renal SAM concentrations were elevated at both 12 and 21 weeks in the liver but GNMT activity only increased in the advanced diabetic condition and there were not concurrent increases in GNMT protein abundance. We have demonstrated the tissue specificity of GNMT regulation previously in response to retinoids (26) as well as the diabetic condition (7). Beyond the increase in

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GNMT activity, there were no observed alternations of enzymes, nor was DNA methylation status affected in the kidney. House et al (28) also found a lack of effect of either glucagon treatment or STZ-diabetes, whereas Jacobs et al (8) reported that renal MS and MTHFR activities were suppressed by a type 1 diabetic condition.

The liver is unique in that it contains the full complement of pathways. The kidney also contains all pathways, although BHMT is expressed at low levels in the rat kidney (30) and MS activity was found not to be quantitatively significant (28,31), making transsulfuration the primary route for removal of homocysteine in the kidney. Homocysteine levels in the ZDF liver were not different from controls at 12 or 21 wks, despite decreased homocysteine concentrations in both the plasma and kidney. The low levels of homocysteine in the kidney are unlikely to be due to increased urinary excretion because this was not shown to be a significant method of homocysteine removal in healthy or STZ-diabetic kidneys (8,31). The kidney has been shown to contribute significantly to the removal of homocysteine and SAH from the circulation (32) and data suggests it contains adequate CBS to compensate for acute or chronic increases in plasma homocysteine (28). Taken together, it could be ascertained that the continued observance of hypohomocysteinemia in rat models might be due to an irreversible loss of homocysteine via the transsulfuration pathway, whereas in human patients homocysteine may also be remethylated to methionine by BHMT in the kidney, thereby conserving the homocysteine moiety and contributing to the development of hyperhomocysteinemia.

The observation of global DNA hypermethylation in the livers of ZDF rats is particularly intriguing. DNA methylation is an important modulator of chromatin structure, repressor of transposable elements, and regulator of gene expression (33,34). DNA is methylated at cytosine residues within the context of CpG dinucleotides. The CpG sequence is underrepresented as only 1-4% of the genome. This is proposed to be due to selection against the sequence due to its high potential for deleterious effects (35). Increasing amounts of 5-methylcytosine increases the chance of point mutations through the genome by virtue of the spontaneous deamination (36). Whereas an unmethylated cytosine base is deaminated to uracil, deamination of methylated cytosine produces thymine. If these altered bases go undetected by DNA repair enzymes prior to replication and the transition mutation will be maintained and carried on to daughter cells. Accumulation of mutations can contribute to genome instability. Another possible explanation for adverse

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effects of genomic hypermethylation might involve hypercondensation of the chromosome. Experimental overexpression of DNMT1 was used to induce genomic DNA hypermethylation and was associated with chromosomal overcondensation, as well as some cases of chromosomal rearrangement and misalignment of sister chromatids (37), which could be expected to alter chromosomal segregation during cell division. Genomic hypermethylation was also associated with increased methylation at histone 3 lysine 9 (H3K9) (37). Interestingly, increased H3K9 dimethylation has also recently been shown in lymphocytes from type 1 diabetic patients and was associated with the promoter regions of many genes of inflammatory processes which may contribute to the development of secondary complications (38). Moreover, one of the few reports of genomic DNA hypermethylation in clinical studies found that leukocytic DNA hypermethylation was associated with increased inflammation (as measured by plasma interleukin-6 concentrations) and increased mortality in patients with chronic kidney disease (39).

In this study, digestion of DNA by restriction enzymes followed by radiolabeled cytosine incorporation provided an assessment of total DNA methylation at the consensus sites for HpaII or BssHII, which represent genomic DNA and CpG island sites respectively. Genomic hypermethylation in this study could lead to genic or chromosomal mutations that would not be detected by the method used in this study. Likewise, differential patterns of methylation in which the overall level of DNA methylation was unchanged would not have been detected. Subsequent studies will be designed to assess DNA methylation in a more site-specific manner.

The pathophysiology of ZDF rats is characterized by obesity, hyperglycemia, hyperinsulinemia, hyperlipidemia, and hypertension (40). ZDF rats develop hyperinsulinemia and insulin resistance before 7 wks of age, with an incremental initial drop in elevated insulin levels around 8 wks of age, blood glucose levels rise and the animals become overtly diabetic by 12 wk of age (41), which we describe here as the early diabetic state. Although insulin levels continue to drop due to the phenomena of pancreatic β-cell exhaustion, ZDF rats may remain hyperinsulinemic relative to lean controls well into the advanced diabetic stage (21), as was also observed in this study. In contrast, as a type 1 models of diabetes, STZ- or alloxan-induced diabetes results in hyperglycemia, but a lack of insulin production due to selective destruction of the pancreatic beta cells. Treatment of rats or hepatic cell lines with glucocorticoids, such as dexamethasone or triamcinolone, has also

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been shown to induce expression of MAT, GNMT, BHMT, and CBS at the transcriptional level (4,11,12,42,43). Insulin administration has been shown to prevent these alterations in both rats and/or cell lines. Interestingly, in healthy individuals insulin stimulates transmethylation, as well as transsulfuration flux (44). Insulin treatment of untreated HepG2 hepatocarcinoma cells was also capable of inducing MAT activity (45). Thus insulin could be seen as playing a role in elevation of SAM concentrations and the metabolic perturbations in the early diabetic state. Separate treatment of HepG2 cells with glucose also resulted in stimulation of MAT activity and furthermore, induced genomic hypermethylation (45). Several recent cell culture studies that were designed to mimic hyperglycemia have shown alteration of histone modification which persist after glucose levels are restored to normal. Based on genome-wide profiling of specific histone modifications, these studies implicate hyperglycemia in the epigenetic regulation of pathways involved in signal transduction, oxidative stress, immune function, and inflammation (38,46-49). The bulk of evidence indicates that aberrations of methyl group metabolism and epigenetic regulation are likely due to the combination of hyperglycemia, a lack of insulin/insulin resistance and/or elevated counter-regulatory hormones. Alternatively, to explain where the response differs with respect to diabetes classification, we might look to c peptide for new insight. In both human patients and animal models, C peptide is increased in type 2 diabetes, but markedly decreased in type 1 diabetes (50-53). C peptide has been shown to be involved in cell signaling – with many insulinomimetic properties - and furthermore, has been implicated in the development of vascular inflammation and atherosclerosis in type 2 diabetes (50). However, the actions of c peptide in type 2 diabetics are largely uncharacterized and may provide an opportunity for investigation into the differences in the pathology of type 1 and type 2 diabetes.

In summary, we have shown that methyl group and homocysteine metabolism was altered in a tissue-specific manner during the progression of type 2 diabetes in the ZDF rat. The response to the diabetic condition and elevated SAM concentrations appears to be more tightly regulated in the liver than the kidney. Although there are many commonalities in the regulation of methyl group and homocysteine metabolism in type 1 and type 2 diabetes, the impact on epigenetic regulation varies between the two conditions. Abnormalities of enzymatic regulation and key metabolite concentrations have been observed as early as 5 weeks of age (9) and we have shown hepatic DNA hypermethylation at 12 weeks of age in ZDF rats. Data are lacking prior to these timepoints, thus it is unclear

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when the abnormalities of methyl group metabolism and epigenetic regulation are initiated in the ZDF rat. However, it is clear that perturbations of methyl group metabolism and aberrant DNA methylation are an early event in the development of a diabetic condition in the ZDF rat with potentially long-lasting effects due to the generally stable nature of epigenetic mechanisms of regulation.

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CHAPTER SIX: Summary and conclusions

General discussion of study results

Our working hypothesis was that sustained induction of glycine *N*-methyltransferase (GNMT), by either retinoids or a diabetic condition, would lead to a functional methyl deficiency, whereby methyl groups would be unavailable for other methyltransferase reactions, such as DNA methylation. We expected that changes in epigenetic regulation, i.e. DNA methylation, would be associated with adverse effects, such as hepatotoxicity. Aberrant epigenetic regulation also represents a possible mechanistic link to hepatocarcinogeneis and the development of the secondary complications of diabetes, which will be addressed in future studies. The three studies presented here studied retinoic acid and diabetes as inducers of GNMT activity and abundance. The induction of GNMT by retinoic acid or diabetes was observed at all measured timepoints, although there were not consistent changes in DNA methylation status, suggesting an influence of other regulatory factors. Methyl group and homocysteine metabolism, as well as epigenetic mechanisms, are intricately tied to health and disease. This information may help in optimization of health or prevention/treatment of disease by laying the foundation for future studies which will assess gene-specific changes in epigenetic regulation and determine the impact of additional regulatory factors.

Here we have demonstrated that induction of hepatic GNMT by all-*trans*-retinoic acid (RA) treatment (3x weekly) was sustained for at least 6 months, but was not associated with changes in DNA methylation status. Although DNA methylation status was unaltered, the possibility remains that other important transmethylation reactions were compromised. However, given the rapid clearance of RA from the plasma and lack of storage in the liver, it is possible that daily RA treatment is necessary for alteration of DNA methylation status and also suggests the possible influence of other regulatory mechanisms in addition to the upregulation of GNMT, which was sustained despite non-daily administration of RA. Furthermore, RA treatment had adverse effects on hematopoiesis and plasma glutathione levels. This research contributes to the growing body of knowledge that will be useful in the risk-benefit analysis of RA therapies.

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In contrast to the response to RA, global DNA methylation status was modulated by type 1 and type 2 diabetes, though the effect was not the same in both diabetic conditions. Despite largely similar patterns of tissue-specific and time-dependent alterations of methyl group and homocysteine metabolism, genomic DNA was hypomethylated in the streptozotocin (STZ)-induced type 1 diabetic rat liver, but genomic DNA hypermethylation was observed in the livers of Zucker (type 2) diabetic fatty (ZDF) rats. These reports of tissue-specific methyl group and homocysteine metabolism, and particularly the association with aberrant DNA methylation status, during the progression of diabetes are novel findings.

Methyl group and homocysteine metabolism in diabetes

The aberrations of methyl group and homocysteine metabolism in diabetic rats are time- and tissue-specific. Hepatic alterations are an early event in both type 1 and type 2 diabetes. In the early diabetic and advanced diabetic condition in the STZ-diabetic rat, we have reported increased hepatic GNMT, phosphatidylethanolamine *N*-methyltransferase (PEMT), betaine-homocysteine *S*-methyltransferase (BHMT), and cystathionine β-synthase (CBS) activity and/or abundance, whereas methionine synthase (MS) activity was decreased. The Brosnan group has reported upregulation of enzymes of all four pathways of hepatic homocysteine metabolism in the prediabetic ZDF rat (1). When data from the studies presented here are taken together with the data of the Brosnan group, it appears that many alterations of methyl group and homocysteine metabolism are present in the early diabetic condition, whereas only a few characteristics are more persistent and are also observed in the advanced diabetic condition, such as the upregulation of hepatic GNMT and BHMT, as well as hypohomocysteinemia.

Hypohomocysteinemia may result in rat models of diabetes due to the lower expression levels of BHMT in the rat liver in comparison to the human liver (2). It has also been shown that methionine synthase and urinary disposal are not major routes for the removal of homocysteine in neither the healthy nor STZ-diabetic rat liver (3,4). However, the kidney has been shown to remove 40% of SAH (5) and at least ~20% of homocysteine from the circulation (6), thereby it must be assumed that in the rat kidney homocysteine is irreversibly catabolized by the transsulfuration pathway, which has been shown experimentally as well (7). In the human kidney, homocysteine may also be remethylated to

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methionine by BHMT, thereby conserving the homocysteine backbone and possibly contributing to the development of hyperhomocysteinemia, particularly in the case of diabetic nephropathy in which transsulfuration flux has been shown to be decreased (8). Although hyperhomocysteinemia has not been observed even in the advanced diabetic conditions in the diabetic rat models used in these studies, downregulation of CBS has been implicated in the development of renal dysfunction in the Dahl salt-sensitive hypertensive rat (9) and both STZ-diabetic and ZDF rats at least had trends towards decreased CBS abundance in the kidney. It is of interest to note that renal homocysteine levels were significantly decreased at 12 wk in the ZDF rats, whereas there was no significant difference in advanced diabetes. Given that signs of renal dysfunction are evidence as early as 14 weeks of age in the ZDF rat (Anderson and Rowling, unpublished observations), this might suggest altered renal handling of homocysteine with the progression of renal disease in this model.

By looking more closely at the data from the ZDF study, we are given greater insight into the roles of the liver and kidney in the early and advanced diabetic conditions (**Figure 1**). The enzymes of the liver appear to be tightly regulated as evidenced by increased GNMT and CBS activity and/or abundance associated with increased intracellular SAM concentration in the early diabetic condition. When hepatic SAM concentrations are similar to controls in the advanced diabetic condition, several methyltransferases are still upregulated at the level of mRNA abundance or protein activity and abundance, but CBS abundance is not different from controls and BHMT mRNA abundance was increased, likely indicating a conservation of methionine. In contrast, the enzymes of methyl group and homocysteine metabolism in the kidney are affected to a lesser extent and metabolite concentrations seem to generally reflect changes in liver and circulation. The only alteration at the transcriptional/translational level noted in the kidney was a trend towards decreased CBS protein abundance in early diabetes. The increase in GNMT activity at 21 weeks was not accompanied by increased GNMT protein abundance, suggesting regulation only at the post-translational level. In the STZ-diabetic rat kidney there was only a transient induction in renal GNMT and CBS protein abundance was decreased. In both the type 1 and type 2 diabetic rat liver, the tissue-specific perturbations of methyl group and homocysteine metabolism preceded or were concurrent with tissue-specific changes in DNA methylation status, with abnormal levels of DNA methylation found in the liver, but not the kidney.

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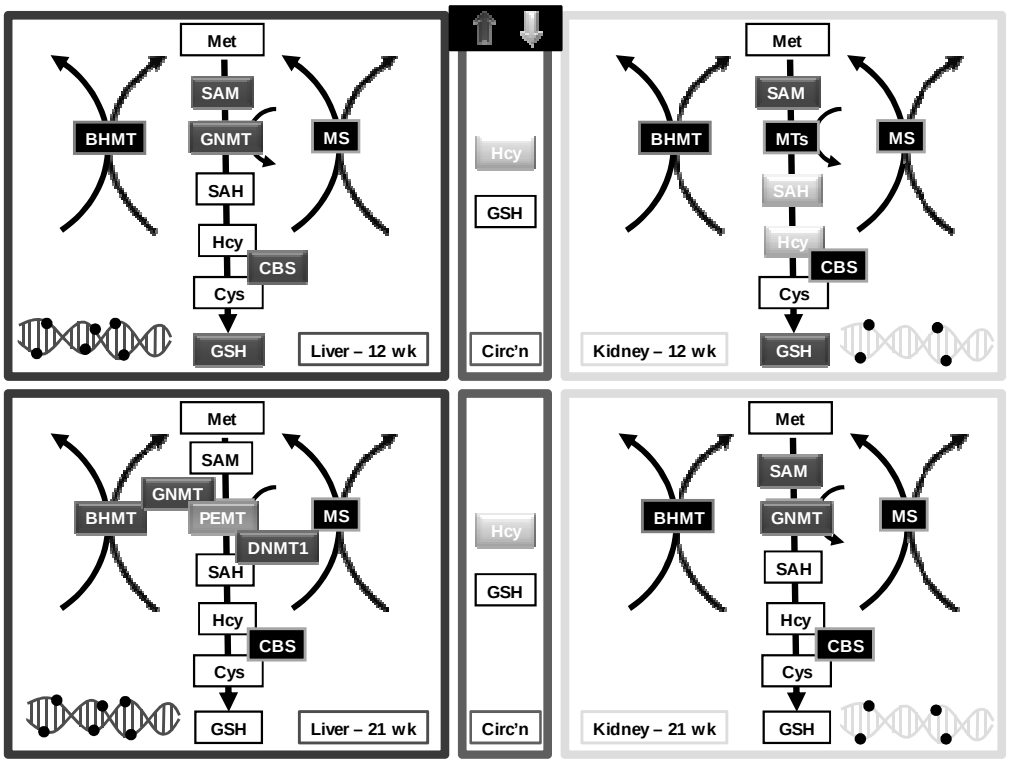

Figure 1. Effects of early (12 wk) and advanced (21 wk) diabetes in the ZDF rat on key regulatory proteins and metabolites of methyl group and homocysteine metabolism

Epigenetic regulation in the diabetic state

For the analysis of epigenetic regulation presented within this work, DNA methylation status was assessed by digestion of DNA with methylation-specific restriction enzymes, followed by cytosine extension. Expression of epigenetic regulatory proteins was estimated by using real-time reverse-transcriptase PCR for determination of relative mRNA abundance of the DNMTs and MBDs. The DNA methylation assay was first published by Pogribny et al (10) and the first published use of the assay by our lab is presented in Chapter 4. This assay was chosen because it was been shown to be sensitive, gives a linear response, and is not affected by DNA damage (though it may be noted that damage was not noted in our isolated DNA when run out on an agarose gel). In the initial workup of the assay the linear response was verified and we have taken care to include appropriate controls as well.

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Digestion of DNA by MspI is not methylation-specific and cleaves at all methylated sites, whereas 3T3 fully-methylated DNA was used as a negative control. Use of these controls verified that the percent methylation for control samples fell within the expected range of 70-90% (11). Use of this method was appropriate for an initial study of DNA methylation status, though it is limited in that it only assesses overall methylation status. We cannot rule out that there may be gene-specific changes in methylation status that are undetected by this assay or that there are changes in methylation patterns, without changing the overall level of CpG island methylation. We anticipate that gene-specific assessment will be the goal of future studies.

Some of the most intriguing findings of these studies were the detection of hypo- and hypermethylation of genomic DNA in the type 1 and type 2 diabetic rat liver respectively. Our lab group has found hepatic genomic DNA hypomethylation in type 1 diabetes is observed not just in the STZ-diabetic rat, but also in the genetic nonobese diabetic mouse model (12). Alterations of genomic DNA methylation status have been associated with increased genomic instability. Genomic DNA hypomethylation is associated with genomic instability due to a more relaxed chromatin structure and fewer repressive mechanisms which facilitates a greater number of recombinational events, movement of transposable elements, loss of imprinting, and overexpression of genes, specifically oncogenes (13). Thus, genomic hypomethylation is a hallmark of cancerous tissue, including hepatocellular carcinomas in rat models and in human patients (13-16). Epidemiological studies support a link between diabetes and cancer as evidenced by an increased incidence of liver cancers of both type 1 and/or type 2 diabetic patients (17-20).

On the opposite end of the spectrum, genomic hypermethylation could increase genomic instability by altering chromatin and overall chromosomal structure, as well as by the accumulation of point mutations. Increasing amounts of methylated cytosine increases the chance of point mutations throughout the genome due to spontaneous deamination (21). Unmethylated cytosine bases are deaminated to uracil, whereas deamination of methylated cytosine produces thymine, a transition mutation. If this TpG-CpG mismatch goes undetected by DNA repair enzymes prior to replication, the transition mutation will be maintained in future cells. Another possible explanation for adverse effects of genomic hypermethylation come from experimental overexpression of DNMT1 which induced genomic DNA hypermethylation and was associated with chromosomal overcondensation,

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as well as some cases of chromosomal rearrangement and misalignment of sister chromatids (22), which could be expected to alter chromosomal segregation during cell division. Genomic hypermethylation was also associated with increased methylation at histone 3 lysine 9 (H3K9) (22). Interestingly, increased H3K9 dimethylation has also recently been shown in lymphocytes from type 1 diabetic patients and was associated with the promoter regions of many genes of inflammatory processes which may contribute to the development of secondary complications (23). Moreover, one of the few reports of genomic DNA hypermethylation in clinical studies found that leukocytic global DNA hypermethylation was associated with increased inflammation (as measured by plasma interleukin-6 concentrations) and increased mortality in patients with chronic kidney disease (24). Thus, both hyper- and hypomethylation of genomic DNA have been associated with adverse clinical outcomes which may be relevant to the diabetic condition.

Possible factors regulating metabolic and epigenetic aberrations in diabetes

Given the relationship between homocysteine metabolism and epigenetic patterns with the pathogenesis of disease, it is critical to determine which factors might play a role in regulating these processes. In the RA-treated rat, it is likely that RA complexed with its receptors acts directly on the GNMT promoter to increase transcription. However, in the diabetic condition, there are numerous potential hormonal influences including glucocorticoid, insulin, incretin hormones and other insulin-related hormones such as c peptide and insulin-like growth factor 1 (IGF-1) which may play a role. Although diabetes perturbations of methyl group and homocysteine metabolism were concurrent with or preceded aberrant DNA methylation levels, when taken together the data from the chronic RA treatment study, it appears that there are also other influencing factors in each of these conditions that contribute to dysregulation of DNA methylation.

A diabetic condition could be described, in part, as hyperglycemia due to a lack of insulin or lack of response to insulin, resulting in a relatively greater effect of glucocorticoids. Similar to the effects of a diabetic condition, treatment of rats or hepatic cell lines with glucocorticoids, such as dexamethasone or triamcinolone, has been shown to increase MAT, GNMT, BHMT, and CBS mRNA abundance, protein abundance, and/or activity levels (25-29). Insulin administration attenuated these effects in both rats and/or cell lines which

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were diabetic or treated with glucocorticoids. However, insulin treatment of HepG2, human hepatocarcinoma cells, cultured in otherwise standard media was also capable of inducing MAT activity (30). Furthermore, in healthy individuals insulin stimulates transmethylation, as well as transsulfuration flux (31). In the short term, this might be expected to protect against the accumulation of homocysteine as proposed by the authors, but if hyperinsulinemia persists, these changes might be described as an early event in prediabetes or a diabetic condition, similar to that observed by the Brosnan group and in our studies of the ZDF rat. However, based on treatment of HepG2 cells, hyperinsulinemia alone was insufficient to induce alterations of DNA methylation status (30). However, glucose treatment increased MAT activity and induced genomic DNA hypermethylation. This suggests a critical role for insulin and/or hyperglycemia in the dysregulation of hepatic methyl group and homocysteine metabolism and it is possible that the period of hyperinsulinemia in the prediabetic state of type 2 diabetes may have a unique influence on metabolic and epigenetic perturbations, though no immediate direct effects on DNA methylation status have yet been observed. This might be further investigated in cell culture by combining hyperinsulinemic and hyperglycemic treatments and determining the impact of concurrent treatment vs. initial treatment with insulin followed by co-treatment to look for additive or synergistic effects. Alternatively, treating healthy rats with insulin could be used to determine the *in vivo* effects on methyl group metabolism and epigenetic regulation.

There are a wide variety of additional factors that are influential in the diabetic conditions including hormonal factors such as glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), insulin-like growth factor-1 (IGF-1) and c peptide. Additionally, many of these parameters are affected by common pharmaceutical and lifestyle interventions for the treatment of diabetes. GLP-1 and GIP are also known as the incretin hormones and both hormones act directly on the pancreas to increase pancreatic beta cell proliferation, insulin synthesis and secretion via direct effects (32). GLP-1 also enhances glucose uptake and storage through effects on the central nervous system. Likewise, IGF-1 has insulin-sensitizing effects, acts directly on the pancreatic beta cells, and additionally, has insulinomimetic effects (33,34). Treatment with IGF-1, agents which decrease the incretin hormones, or agonists of incretin receptors are the focus of many anti-diabetic therapies, which are often successful in slowing the progressing of type 2 diabetes and minimizing complications (32-35). However, these medications do not work indefinitely and do not abrogate all metabolic perturbations of type 2 diabetes (35). Studies

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of insulin-sensitizing interventions in the ZDF rat show a similar pattern. An exercise intervention consisting of 8 wk of treadmill running was successful in improving insulin sensitivity and preventing increases in blood glucose in the ZDF rat at 14 wk of age (36). However, fasting serum insulin levels continued to rise slowly in ZDF rats despite the exercise intervention, which might suggest the course of the disease was only delayed. The effect of exercise on methyl group and homocysteine metabolism was not determined in this study. However, in the insulin resistant (not diabetic) Zucker fatty rat, three weeks of troglitazone treatment increased hepatic CBS activity which was proposed to have contributed to the observed decrease in plasma homocysteine, but the hepatic SAM:SAH ratio was decreased relative to untreated Zucker fatty rats (37). This data from the strain from which the ZDF rat was selectively bred suggests that insulin-sensitizing drugs may modulate methyl group and homocysteine metabolism in the prediabetic condition, though this limited evidence does not fully explain what effect these alterations may have on transmethylation reactions. Troglitazone works as an activator of PPARα and γ, suggesting the possible involvement of yet another regulatory pathway. Whereas the factors discussed thus far affect beta cell function and insulin action specifically, another possibility lies in c peptide which, like insulin, is differentially regulated in type 1 and 2 diabetic conditions and also has independent cellular signaling mechanisms (38).

Future research

There are several areas towards which future research efforts could be directed. Here are four potential aims: i) perform a more in-depth study of perturbed epigenetic regulation in type 1 and type 2 diabetes, ii) investigate possible factors involved in the different epigenetic response in advanced type 1 and type 2 diabetic conditions, iii) define the mechanisms by which modulators of methyl group and homocysteine exert their effects, and iv) develop dietary therapies for optimal health, prevention of disease, or treatment of adverse effects.

The results from the studies presented herein indicate the presence of diabetes-induced alterations of DNA methylation status. Thus far we have shown this as overall net changes in DNA methylation, but to gain a greater understanding of the biological significance of said aberrations will require localization of the sites at which methylation

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status is changed. This information will be crucial for linking alterations of methyl group metabolism and epigenetic regulation to the development of secondary complications of diabetes, which was not addressed by the current studies. To accomplish this, experiments should be designed to assess both specific changes in epigenetic regulation as well as the progression of secondary complications of diabetes.

For the determination of specific alterations of epigenetic regulation by DNA methylation the most comprehensive approach would use of genome-wide analyses such as the Methyl-Seq technique, which utilizes next-generation sequencing tools, or DNA methylation-specific microarray. Each technique offers advantages: Methyl-Seq offers a nonbiased analysis and will analyze changes in DNA methylation at both genic and nongenic sites (39), whereas microarrays are also available for the entire genome (40) or specifically covering all known promoter sites for genes and miRNAs, as well as all CpG islands (41), which may help to focus in on the most biologically-relevant changes. For the study of individual genes, bisulfite treatment and methylation-specific PCR techniques could be used. Based on recent genome-wide analyses of histone modifications in cultured and primary cells from diabetics which revealed widespread and gene-specific alterations of histones (23,42-45), as well as our data which demonstrated changes in overall DNA methylation levels, we anticipate that we will likewise find changes in DNA status at specific sites within the genome. Alterations of DNA methylation status are expected in pathways involved in signal transduction, oxidative stress, immune function, and inflammation. Notably, we detected a transient change in methylation status in the CpG islands in the STZ-diabetic rat; it is possible that a similar transition occurred in the ZDF rat, but was not captured by our study based on the timepoints at which we collected our samples.

The development of secondary complications and their relationship to epigenetic alterations observed in diabetic conditions will require more thorough assessments of kidney and vascular function. Our studies used basic assessments of renal dysfunction by comparing relative renal weights and plasma creatinine concentrations. Although these methods are have been used by others in diabetic rats (46,47), renal dysfunction might be more adequately assessed in future studies by histological examination and measurement of additional circulatory and urinary substances such as has been performed previously in diabetic rat models (47). Plasma and urinary levels of hemoglobin, albumin, and creatinine, as well as plasma cystatin c would all be appropriate biochemical measures (46-48).

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Plasma cystatin c is a more recently established biomarker of renal dysfunction that has the additional advantage of not being affected by muscle mass or malignancy and would be expected to be appropriate for use in a diabetic population (49,50). Histochemistry and immunohistochemistry methods utilizing hematoxylin and eosin y staining, alone or in combination with antibodies against transforming growth factor-β, could be utilized for the assessment of alterations of glomerular structure and detection of renal fibrosis. For future studies of cardiovascular disease, we have proposed to assess the progression of atherosclerosis as indicated by the vascular response to vasodilators (51), time to thrombosis following injury (52), and platelet coactivation activity (53). In similar studies of ZDF rats, early indicators of vascular and renal dysfunction are noted around 14-16 weeks of age (54, Anderson and Rowling, unpublished observations) and at necropsy we noted gross morphological abnormalities in the kidneys of ZDF rats in the advanced diabetic state.

The second and third aims relate to increasing understanding of the specific factors that are known to alter methyl group metabolism and epigenetic regulation. The divergent regulation of DNA methylation status by type 1 and type 2 diabetic conditions is an intriguing finding and efforts should be made to determine possible mediators of this effect. As has been previously discussed, one possibility lies in the timing of insulin exposure based on evidence that insulin attenuates abnormal methyl group and homocysteine metabolism in type 1 diabetic models and patients (8,27,28,55, Nieman and Schalinske, unpublished observations), whereas hyperinsulinemia in healthy individuals and otherwise untreated cell culture models was associated with increased transmethylation and transsulfuration (30,31). At the surface, it appears that the effect of c peptide might also be a promising target of investigation because it is differentially expressed in type 1 and type 2 diabetes, has some insulin-like effects, but is also biologically active by a separate receptor, and its functions are likely not yet fully characterized (38). We, and others, have shown that many other hormones or biologically active compounds have a profound impact on methyl group and homocysteine metabolism (56). Studies addressing the mechanisms by which these compounds - largely ligands for nuclear receptors such as thyroid hormones, retinoids, glucocorticoids – exert their regulatory effects may help provide for the development of a more unified theory of regulation of methyl group and homocysteine metabolism.

Lastly, it would be desirable to be able to formulate some form of dietary therapy to ameliorate the effects of perturbed methyl group metabolism. Methyl group

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supplementation might be used in type 1 diabetes with the goal of providing adequate levels of methyl groups for all SAM-dependent transmethylation reactions. When considering supplementation of methyl groups there are many combinations of donors and cofactors that could be provided; however, the most efficient approach would be to use an inclusive mixture that has been shown to be optimally effective in previous studies (57). Thus, supplementation groups might receive a modification of our standard semi-purified AIN93 diet containing (per kg diet): methionine, 3g; choline, 5 g; betaine, 5 g; folate, 5 mg; B_{12}, 0.5 mg; and B_6, 10 mg (57,58). A number of studies have also shown that SAM can be provided via the diet and is bioavailable in both rats and humans (59-61). It is also of interest to note that SAM has recently been reported to increase insulin sensitivity (62) and this compound is available over-the-counter. This makes it an attractive option based on consumer availability and also lends particular importance to gaining a greater understanding of the effects of taking such a supplement since many individuals may already be using this product.

Another possibility for dietary intervention might be through altering methionine and cysteine content. We have previously shown that methionine supplementation or feeding of egg white protein (a protein with a higher methionine:cysteine ratio than the casein standard) can alter methyl group and homocysteine metabolism (63,64). Feeding excess methionine (10% casein plus 0.3% vs. 0.5-2.0% methionine) results in a dose-dependent increase in hepatic GNMT (63) and it has long been known that excess methionine and SAM stimulates transsulfuration (65). When egg white protein was substituted for casein, hepatic GNMT and BHMT activities were increased and plasma homocysteine was decreased (64). Given this upregulation of specific transmethylation reactions and transsulfuration in response to increased methionine or methionine:cysteine ratio, it stands to reason that feeding diets with a protein with a low methionine:cysteine ratio or by supplementing cysteine, the drive towards increased transmethylation and transsulfuration might be reduced. A recent study shows promise for this approach given that 8 wks of L-cysteine supplementation lowered blood glucose levels and inflammatory markers in ZDF rats relative to saline-treated controls (66). Plasma glutathione levels were unchanged between groups, but we have not shown this to be altered in the ZDF rat and data was not collected on any other key regulatory proteins of metabolites of methyl group or homocysteine metabolism. This hypothesis certainly warrants future investigation.

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In addition to the differences in methyl group utilization, specifically with regards to DNA methylation, between the type 1 and type 2 conditions, challenges to implementation of dietary therapies include timing effects and confounding factors such as dietary intake and enzyme polymorphisms. The timing of dietary intervention has been shown to be particularly important, especially with regards to influencing epigenetic mechanisms. DNA methylation and other forms of epigenetic regulation seem to be particularly vulnerable to change during the perinatal and pubertal periods and in aging (67,68). Likewise, it appears that in the progression of disease, it is likely that there is only a specific window of time in which the effects may be prevented or reversed, as has been shown in the methyl-deficient diet model of hepatocarcinogenesis (69). Furthermore, in human populations, the response of homocysteine and folate one-carbon metabolism to dietary interventions has been shown to be affected by polymorphisms within these pathways (70-72)

Overall conclusions

In summary, these studies have demonstrated the changes of methyl group and homocysteine metabolism in long-term intermittent retinoid treatment, as well as during the progression of type 1 and type 2 diabetic conditions. Furthermore, we have characterized perturbations of overall epigenetic regulation by DNA methylation in a diabetic condition. In the diabetic conditions, alterations in methyl group and homocysteine metabolism preceded or were concurrent with changes in epigenetic regulation. Although GNMT was upregulated by retinoid treatment and in both diabetic conditions, the effects on DNA methylation status varied. This disparity suggests a role for additional factors impacting epigenetic regulation. Future studies should be conducted with the goal of elucidating which factors are most important in the modulation of methyl group and homocysteine metabolism, as well as regulation of DNA methylation patterns. It will also be of the utmost interest to determine the specific nature of the epigenetic alterations observed here and greater efforts must be made to identify potential linkages between these changes and the development of diabetic complications, thus providing insight into the biological significance of abnormal methylation in diabetes and potentially providing targets for future therapies.

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APPENDIX A. Chapter 4 Supplemental Data

Plasma creatinine was significantly decreased in the STZ-diabetic rat at 8 wk.

	Plasma creatinine (mg/dL)		
Time (weeks)	Control	STZ-Diabetic	p-value
2	1.71 ± 0.19	1.60 ± 0.22	0.712
4	2.08 ± 0.23	1.45 ± 0.19	0.065
8	2.28 ± 0.30	0.88 ± 0.15*	0.015

Data are means ± SEM (n = 5). *indicates significantly different from control, $p < 0.05$.

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Hepatic DNA is hypomethylated, whereas there were no significant differences in renal DNA methylation status

	Liver			Kidney		
Time (weeks)	**Control**	**STZ-diabetic**	**p-value**	**Control**	**STZ-diabetic**	**p-value**
Global methylation, *3H-dCTP incorporation (DPM/µg DNA)*						
2	4476 ± 1325	6996 ± 604	0.134	8709 ± 757	8741 ± 461	0.971
4	4053 ± 493	6505 ± 1088	0.074	8869 ± 278	9622 ± 695	0.338
8	4827 ± 349	8137 ± 753*	0.004	7854 ± 634	7553 ± 889	0.785
CpG island methylation, *3H-dCTP incorporation (DPM/µg DNA)*						
2	237 ± 17	437 ± 35*	<0.001	781 ± 104	521 ± 105	0.128
4	270 ± 32	324 ± 24	0.214	1017 ± 240	551 ± 234	0.238
8	276 ± 15	330 ± 34	0.195	1273 ± 206	1032 ± 277	0.508

Data are means ± SEM. *indicates significantly different from control, $p < 0.05$.

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APPENDIX B. Chapter 5 Supplemental Data

Body weights of lean and ZDF rats in the study of advanced diabetes. Data are means, statistical analysis at 21 wk presented in Table 2 within the text.

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Hepatic mRNA abundance of key proteins of epigenetic regulation was unchanged at 12 wk in ZDF rats compared to lean controls

	Mean fold induction		
Target	Lean	ZDF	p-value
DNMT1	1.00 ± 0.23	0.97 ± 0.33	0.944
DNMT3a	1.00 ± 0.66	0.20 ± 0.10	0.215
DNMT3b	1.00 ± 0.44	0.33 ± 0.13	0.150
MBD1	1.00 ± 0.51	0.60 ± 0.32	0.504
MBD2	1.00 ± 0.73	0.18 ± 0.06	0.247
MBD3	1.00 ± 0.68	0.17 ± 0.11	0.213
MBD4	1.00 ± 0.70	0.11 ± 0.09	0.247
MeCP2	1.00 ± 0.65	0.15 ± 0.08	0.429

Data are means ± SEM, * indicates significant difference, $P < 0.05$

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