Type I Diabetes Leads to Tissue-Specific DNA Hypomethylation in Male Rats

Kelly T. Williams, Timothy A. Garrow, and Kevin L. Schalinske

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 the 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 and 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 weight) 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 kidney did not differ. 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 time points. Most importantly, we have shown 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.


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 2 factors: dietary methyl intake and methyl group utilization. The major dietary methyl donors are methionine, choline, and betaine (5). Production of S-methyltetrahydrofolate from S-methyltetrahydrofolate via methylenetetrahydrofolate reductase (MTHFR) serves as an endogenous methyl donor. Methyl group metabolism consists of 4 processes: transmethylation, remethylation by folate/vitamin 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 vitamin B-12-dependent enzyme methionine synthase (MS) utilizes a methyl group from S-methyltetrahydrofolate. 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 phosphatidylethanolamine 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 incor
poration 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 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 decreased (9,11), suggesting the potential development of methyl deficiency. Furthermore, both the activity of MTHFR in lymphocytes and the intracellular SAM:SAH ratio, 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: [14C-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-[14C]-methyl-THF, Amersham Pharmacia; goat anti-mouse horseradish peroxidase secondary antibody Southern Biotechnology; phenylmethylsulfonyl fluoride, Calbiochem; and S-adenosyl-L-[3H]-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. 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 under a 12-h light-dark cycle. All rats consumed ad libium a semipurified diet (17) and were prepared using the method of Fell et al. (24) and SAM and SAH were 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 antibody. Densitometric analysis (SigmaGel Software, SPSS) 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; 20 min at 4°C, the supernatant was removed and centrifuged at 100,000 × g; 60 min at 4°C. The resulting microsomal pellet was 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-vitamin B-12-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 mono-potassium phosphate (20). As described previously (7), BHMT and MS activities were assessed by radioisotopic assays utilizing [methyl-14C]-betaine (20) and [methyl-14C]-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 C18 Radial-Pak column (Waters) using a mobile phase of 40 mL/L acetonitrile in 0.1 mol/L potassium phosphate buffer (pH 2.1). The addition of N-acetylcycteine (1 mmol/L) to each sample prior to derivatization 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 HpaII and Bsu36I (New England Biolabs) for determination of global and CpG island methylation, respectively. For the cytosine extension assay, a reaction mixture of the DNA digest, 10× PCR Buffer II (without MgCl2), 25 mmol/L MgCl2, 0.5 U AmpliTaq DNA Polymerase (Applied Biosystems), and [3H]-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) 3 times, dried, and 3H incorporation was assessed using liquid scintillation counting.

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Diabetes-induced hepatic DNA hypomethylation 2065
**Results**

**Methyl group metabolism is perturbed by diabetes.** Blood glucose levels were greater in diabetic rats than control rats at all time points (72, 151, and 185% greater than control at wk 2, 4, and 8, respectively; Table 1). Plasma homocysteine concentrations were lower in the diabetic group at all time points (76, 65, and 53% of control at wk 2, 4, and 8, respectively; Table 1).

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 with 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 diabetic rats was lower than controls, but there was no significant change in MS activity. There was a sustained increase in hepatic CBS abundance in diabetic rats, with an increase of 35% at 8 wk (Fig. 1). In contrast, renal CBS abundance decreased by 33% at 8 wk in the diabetic group compared with controls, which would also be expected to 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 time points (Fig. 2A), although the magnitude was diminished with time. The temporal pattern of GNMT induction by diabetes was tissue specific. Renal GNMT activity was elevated at 2 and 4 wk in diabetic rats but did not differ from control values at 8 wk (Fig. 2B). Likewise, renal GNMT abundance was greater only at the 2 wk time point in diabetic rats.

**DNA methylation status is affected by a diabetic condition in the liver but not in the kidney.** DNA methylation status was assessed using the cytosine extension DNA methylation assay (25), wherein endogenous DNA hypomethylation is indicated by an increase in [3H]dCTP incorporation. For liver, genomic DNA methylation did not differ between control and diabetic rats at 2 wk. However, there was a trend toward greater [3H]dCTP incorporation in diabetic rats at 4 wk (P = 0.074) and an increase of 70% in diabetic rats compared with controls at 8 wk (Fig. 3; P = 0.004). Hepatic Cpg island DNA was hypomethylated in diabetic rats at 2 wk; however, Cpg island methylation status did not differ at 4 or 8 wk (data not shown). 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 time points combined (r = 29, r = 0.74; P < 0.01). In marked contrast to hepatic tissue, renal DNA methylation status did not differ at any time point. Taken together, this data suggests that perturbations of hepatic methyl group metabolism by a diabetic con-

**TABLE 1** Circulating concentrations of glucose and homocysteine in control and diabetic rats

<table>
<thead>
<tr>
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<th>Week</th>
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<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>8</td>
<td></td>
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<tr>
<td>Blood glucose, mmol/L</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Control</td>
<td>12.2±0.3</td>
<td>9.7±0.5</td>
<td>9.8±0.7</td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td>21.0±1.7*</td>
<td>24.4±2.8*</td>
<td>27.9±1.1*</td>
<td></td>
</tr>
<tr>
<td>Plasma homocysteine, μmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>9.1±0.3</td>
<td>10.0±2.0</td>
<td>7.4±0.8</td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td>2.2±0.2*</td>
<td>3.5±0.5*</td>
<td>3.5±0.6*</td>
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1 Values are means ± SEM, n = 4–5. *Different from control at a time, P < 0.05.

**TABLE 2** Hepatic and renal remethylation enzyme activity levels in control and diabetic rats at 8 wk after induction of diabetes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHMT activity, nmol Met/(h - mg protein)</td>
<td>73±10</td>
<td>142±16*</td>
</tr>
<tr>
<td>Renal MS activity, pmol/(min - mg protein)</td>
<td>0.3±0.1</td>
<td>1.1±0.1*</td>
</tr>
<tr>
<td>Hepatic</td>
<td>24.0±2.9</td>
<td>4.5±1.6*</td>
</tr>
<tr>
<td>Renal</td>
<td>209±28</td>
<td>148±24</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 4–5. *Different from control, P < 0.05.
dition were sustained through 8 wk, with subsequent alterations of DNA methylation status and elevated DNMT1 abundance, whereas the kidney appears to be less sensitive.

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 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 that showed that 10 wk after induction of diabetes, plasma homocysteine concentrations remained lower in diabetic rats than in 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, diabetic hyperfiltration was present, as evidenced by lower plasma creatinine concentrations (data not shown). Typically, plasma homocysteine and creatinine concentrations are inversely related to the glomerular filtration rate; thus, during the hyperfiltration that occurs in early diabetes in both humans and animal models, hypohomocysteinemia is observed (31–33). However, plasma homocysteine and creatinine concentrations were not correlated ($r = 0.279$; $P = 0.15$), 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 DNA methylation did not change. 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 DNMT1 and methyl-binding proteins, aberrant histone modifications, uracil misincorporation, and DNA stands 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.

The results presented here suggest that alterations of methyl group metabolism were sustained in the liver, whereas the kid-

### TABLE 3

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

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM, nmol/g liver</td>
<td>28.0 ± 4.5</td>
<td>22.3 ± 5.8</td>
</tr>
<tr>
<td>SAH, nmol/g liver</td>
<td>4.8 ± 0.5</td>
<td>5.5 ± 0.8</td>
</tr>
<tr>
<td>SAM/SAH</td>
<td>6.4 ± 1.4</td>
<td>4.6 ± 1.4</td>
</tr>
<tr>
<td>DNMT1 abundance, fold of control</td>
<td>1.00 ± 0.16</td>
<td>1.46 ± 0.05*</td>
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1 Values are means ± SEM, $n = 4–5$. *Different from control, $P < 0.05$.  

![FIGURE 2](https://academic.oup.com/jn/article-abstract/138/11/2064/4670000)  
![FIGURE 3](https://academic.oup.com/jn/article-abstract/138/11/2064/4670000)
ney 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, have been previously described in acute models of type 1 and/or 2 diabetes (6–12). The data presented here suggest 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 is similar to the methyl-deficient rat model, such that hepatic methyl group and methionine metabolism is perturbed for at least 8 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 because its expression is limited to gluconeogenic tissues (38); it is also a proposed regulator of the SAM:SAH ratio (39). Therefore, 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 ratio in diabetic rats in this study did not differ, 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 that 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 methyl-ation capacity (48). GNMT is proposed to be 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 Martínez-Chantar et al. (49) demonstrates that methylation status of specific genes was increased in GNMT-knockout mice. 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 that 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 that chronic alterations of methyl group metabolism concomitant with genomic hypomethylation in the rat liver are 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 toward 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.

**Literature Cited**


