Insulin administration abrogates perturbation of methyl group and homocysteine metabolism in streptozotocin-treated type 1 diabetic rats

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Nieman KM, Schalinske KL. Insulin administration abrogates perturbation of methyl group and homocysteine metabolism in streptozotocin-treated type 1 diabetic rats. Am J Physiol Endocrinol Metab 301: E560–E565, 2011. First published July 5, 2011; doi:10.1152/ajpendo.00105.2011.—Modifications in methyl group and homocysteine metabolism are associated with a number of pathologies, including vascular disease, cancer, and neural tube defects. A diabetic state is known to alter both methyl group and homocysteine metabolism, and glycine N-methyltransferase (GNMT) is a major regulatory protein that controls the supply and utilization of methyl groups. We have shown previously that diabetes induces GNMT expression and reduces plasma homocysteine pools by stimulating both its catabolism and folate-independent remethylation. This study was conducted to determine whether insulin plays a role in the control of homocysteine concentrations and GNMT as well as other key regulatory proteins. Male Sprague-Dawley rats were randomly assigned to one of three groups: control, streptozotocin (STZ)-induced diabetic (60 mg/kg body wt), and insulin-treated diabetic (1.0 U bid). After 5 days, rats were anesthetized (ketamine-xylazine) for procurement of blood and tissues. A 1.5-fold elevation in hepatic GNMT activity and hypohomocysteinemia in diabetic rats was completely prevented by insulin treatment. Additionally, diabetes-mediated alterations in methionine synthase, phosphatidylethanolamine N-methyltransferase, and DNA methylation were also prevented by insulin. We hypothesize that the concentration of blood glucose may represent a regulatory signal to modify GNMT and homocysteine. In support of this, blood glucose concentrations were negatively correlated with total plasma homocysteine (r = −0.75, P < 0.001) and positively correlated with GNMT activity (r = 0.77, P < 0.001). Future research will focus on further elucidating the role of glucose or insulin as a signal for regulating homocysteine and methyl group metabolism.

diabetes; methyl group metabolism; DNA methylation

METABOLISM OF METHYL GROUPS AND HOMOCYSTEINE are critical pathways in the optimization of health and prevention of disease. The primary methyl donor S-adenosylmethionine (SAM) requires a constant supply of methyl groups for numerous transmethylation reactions (Fig. 1), including the biosynthesis and modification of nucleic acids, proteins, and lipids (36). Methionine, provided through the diet or endogenously via homocysteine remethylation, is activated by methionine adenosyltransferase to form SAM. Epigenetic modification of DNA by methylation requires methyl groups from SAM and is a critical mechanism in regulating tissue-specific gene expression as well as genome function (26). This reaction is catalyzed by a family of DNA methyltransferases that transfer a methyl group from SAM to CpG dinucleotides. Aberrant DNA methylation is associated with numerous pathologies, including cancer development and vascular disease (4, 8, 14, 15, 29).

Clearly, abnormal regulation of methyl group metabolism is a major determinant in the pathogenesis of a number of diseases, and glycine N-methyltransferase (GNMT) is a key regulatory protein. GNMT is an abundant cytosolic protein that functions to optimize the SAM/S-adenosylhomocysteine (SAH) ratio by transferring methyl groups from SAM to glycine and generating sarcosine. The SAM/SAH ratio is an indicator of cellular transmethylation potential; SAH is a potent inhibitor of most transmethylation reactions, whereas GNMT is only weakly inhibited (3, 17). Moreover, GNMT activity is allosterically inhibited by the folate coenzyme 5-methyltetrahydrofolate (5-CH3THF), and SAM is a negative allosteric regulator for 5,10-methylenetetrahydrofolate reductase (MTHFR), the enzyme responsible for 5-CH3THF production (13, 18). Thus, excess methyl groups in the form of SAM inhibit MTHFR activity and 5-CH3THF synthesis, thereby alleviating its inhibition on GNMT activity, resulting in the disposal of excess methyl groups in the form of sarcosine. Alternatively, under methyl-deficient conditions, the inhibition of MTHFR by SAM is alleviated, thereby increasing 5-CH3THF synthesis and subsequently inhibiting GNMT activity as a means to conserve methyl groups for the most essential transmethylation reactions (37). Hepatic expression and function of phosphatidylethanolamine N-methyltransferase (PEMT) is also a key enzyme in the regulation of methyl group and homocysteine metabolism. PEMT catalyzes the conversion of phosphatidylethanolamine to phosphatidylcholine via the sequential addition of three methyl groups from SAM and has been proposed to represent the largest consumer of SAM-derived methyl groups as well as function to regulate homocysteine balance (32).

Homocysteine is a non-protein-forming amino acid that is a product of all SAM-dependent transmethylation reactions following the action of SAH hydrolase. Homocysteine must be either remethylated back to methionine or irreversibly catabolized through the transsulfuration pathway to cysteine, a pathway that is dependent on two B6-dependent enzymes, cystathionine β-synthase (CBS) and γ-cystathionase. Homocysteine remethylation occurs by both folate-dependent and -independent mechanisms, which have been reported to contribute equally to the remethylation of homocysteine (5). Folate-dependent remethylation requires donation of a methyl group from 5-CH3THF in a reaction catalyzed by the B12-dependent enzyme methionine synthase (MS). Betaine, derived from the oxidation of choline, is the methyl group donor in folate-
increased cystathionine treated with glucocorticoids and glucagon, owing in part to humans and rats as well as in reports of animals and cells complications, hypohomocysteinemia has been found in both that results in the perturbation of homocysteine and methyl group metabolism by disrupting the expression of key regulatory proteins and potentially linking them to associated pathologies have been identified. A diabetic state, characterized by counterregulatory hormones, has been shown to be a condition of BHMT.

Several factors that influence homocysteine and methyl group metabolism by disrupting the expression of key regulatory proteins and potentially linking them to associated pathologies have been identified. A diabetic state, characterized by reduced insulin levels, hyperglycemia, and elevated circulating counterregulatory hormones, has been shown to be a condition that results in the perturbation of homocysteine and methyl group metabolism. For both type 1 and 2 diabetes without renal complications, hypohomocysteinemia has been found in both humans and rats as well as in reports of animals and cells treated with glucocorticoids and glucagon, owing in part to increased cystathionine β-synthase and γ-cystathionase activity (1, 10, 11, 21, 24, 27, 30, 38). Folate-independent remethylation may also play a role in diminished homocysteine concentrations, owing to an increase in the expression and function of BHMT (21, 25, 38). However, renal function declines during the pathogenesis of diabetes, the circulating concentrations of homocysteine rise (9, 23).

We have shown that a type 1 diabetic state induced by streptozotocin (STZ) administration results in a significant disruption in the metabolism of methyl groups and homocysteine, characterized by the hepatic induction of GNMT, PEMT, and BHMT, combined with a decrease in MS activity and plasma homocysteine concentrations (7, 20, 21). Moreover, these collective alterations have been shown to lead to global DNA hypomethylation (39), an important epigenetic mark in gene regulation, as well as a potential link between diabetes and its complications. A significant question is whether these observations are the result of high glucose or a lack of insulin and/or whether they are secondary to using a chemically induced model of type 1 diabetes. Understanding the signals that alter methyl group metabolism is a key component towards developing strategies for diabetes prevention and/or treatment.

**MATERIALS AND METHODS**

**Chemicals.** Reagents were obtained from the following sources: S-adenosyl-L-[methyl-³H]methionine (PerkinElmer Life Sciences), chemiluminescent Western blotting detection reagents (GE Healthcare), streptozotocin and S-adenosyl-L-methionine (Sigma-Aldrich), goat anti-mouse IgG horseradish peroxidase (Southern Biotechnology), HpaII and BssHII endonuclease (New England Biolabs), and [³H]-dCTP (NEN Life Science Products). The GNMT antibody was generously provided by Dr. Yi-Ming Chen of the National Yang-Ming University, Taipei, Taiwan (19). All other chemicals were of analytical grade.

**Animals and diets.** All animal protocols were approved by and conducted in accordance with guidelines set forth by the Iowa State University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (125–149 g) were housed individually in plastic cages in a room with a 12:12-h light-dark cycle. Animals were allowed access to food and water ad libitum. Following a 6-day acclimation period on a control diet (30), rats were randomly assigned to one of three treatment groups: control, diabetes (STZ), or diabetes treated with insulin (STZ + INS). Following the acclimation period, rats received a single intraperitoneal injection of STZ (60 mg/kg body wt) or vehicle (10 mM citrate buffer, pH 4.5). After 24 h, rats assigned to the STZ + INS group were administered insulin (porcine pancreas, 1.0 U bid in 200 µl of saline) intraperitoneally every 12 h (6) or vehicle for a total of 5 days. On day 6, all rats were anesthetized with a mixture of ketamine-xylazine (90:10 mg/kg body wt), and heparinized whole blood was collected by cardiac puncture. An aliquot was removed for the determination of blood glucose concentrations using a commercial kit (Sigma-Aldrich). Diabetes was defined as a blood glucose concentration >20 mM. The remaining whole blood was centrifuged at 5,000 g for 5 min, and the resulting plasma was stored at −20°C for subsequent determination of homocysteine concentrations. Portions of the liver were rapidly removed and homogenized in ice-cold phosphate-buffered (10 mM, pH 7.0) sucrose (0.25 M) containing 1 mM EDTA, 1 mM sodium azide, and 0.1 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 20,000 g for 30 min, and an aliquot of the resulting supernatant was stored at −70°C for the determination of GNMT activity and abundance as well as MS activity. For the determination of PEMT activity, an additional homogenate aliquot was centrifuged at 100,000 g, and the
resulting microsomal pellet was resuspended in 0.25 M sucrose buffer and stored at \(-70^\circ\text{C}\). Additional portions of the liver were collected and snap-frozen in liquid nitrogen for DNA isolation and methylation analysis. Total soluble protein concentrations of the cytosolic and microsomal fractions were determined using a commercial kit (Coomassie Plus; Pierce) and bovine serum albumin as a standard.

**Enzyme analysis.** The enzymatic activity of GNMT was determined using a radioisotopic assay, as reported previously (30). GNMT protein abundance was quantified by immunoblotting techniques using a 10–20% gradient SDS-polyacrylamide gel to quantify the 32-kDa monomer subunit of GNMT (30). Following electrophoretic separation, proteins were transferred to a nitrocellulose membrane and incubated overnight at 4°C with a 1:5,000 dilution of a monoclonal anti-mouse IgG horseradish peroxidase secondary antibody, and GNMT antibody (19). The membrane was then incubated with goat anti-mouse IgG horseradish peroxidase secondary antibody, and GNMT protein abundance was visualized using chemiluminescence detection and exposure to Kodak X-Omat AR film. Densitometric analysis was performed using SigmaGel software (SPSS). The activity of MS was determined by a radioisotopic assay using \[^{[14]}\text{CH}_3\]THF, as described previously (16, 20, 21). PEMP activity was determined by measuring the in vitro incorporation of methyl groups into S-adenosyl-L-[methyl-\(^{[3]}\text{H}\)]methionine into phospholipids (7).

**Homocysteine analysis.** Total plasma homocysteine concentrations were determined using HPLC with fluorescence detection (34). Plasma samples were derivatized with 0.1% (\text{v/vol}) 4-fluoro-7-sulfobenzo-furazan (ammonium salt), and N-acetylcysteine (final, 1 mM) was added to the plasma samples as an internal standard. Aliquots were injected onto a Bondapak C\(_18\) Radial-Pak column (Waters) equilibrated in a mobile phase consisting of 4% acetonitrile in 0.1 M potassium phosphate buffer (pH 2.1).

**Determination of DNA methylation.** DNA methylation status was assessed by measuring the in vitro incorporation of methyl groups into CpG dinucleotides, as described by Pogribny et al. (22) with minor modifications (39). Hepatic DNA was isolated from liver samples using a commercial kit (Promega), followed by overnight digestion using HpaII and BssHII endonuclease (New England Biolabs) for global CpG and CpG island analysis, respectively. The methylation assay mixture consisted of 1.0 \(\mu\text{g}\) of digested DNA, 1 \(\times\) PCR Buffer II, 1 mM MgCl\(_2\), 0.5 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), and 9.3 \(\mu\text{M}\) \[^{[3]}\text{H}\]dCTP in a total volume of 40 \(\mu\text{L}\). Following a 1-h incubation period at 55°C, samples were applied to DE-81 ion exchange filter paper, washed with 0.5 M sodium phosphate buffer, and dried, and \[^{[3]}\text{H}\]dCTP incorporation was determined by liquid scintillation counting. The extent of \[^{[3]}\text{H}\]dCTP incorporation is inversely proportional to the degree of endogenous methylation (22).

**Statistical analysis.** The mean values of each treatment group were analyzed using a one-way ANOVA test. When the results across treatment groups were significantly different (\(P < 0.05\)), mean values were compared using Fisher’s least significant difference procedure. GNMT activity, PEMT activity, total homocysteine, and blood glucose concentrations were subjected to a Pearson correlation analysis (31). All statistical analysis was performed using SigmaStat software.

### Table 1. Cumulative weight gain, blood glucose concentrations, and plasma homocysteine concentrations in control and STZ-induced diabetic rats with or without INS treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight Gain, g</th>
<th>Blood Glucose, mM</th>
<th>Plasma Homocysteine, (\mu\text{M})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>60 ± 2</td>
<td>9.0 ± 1.0</td>
<td>4.9 ± 0.7</td>
</tr>
<tr>
<td>STZ</td>
<td>29 ± 4*</td>
<td>26.1 ± 1.3*</td>
<td>2.1 ± 0.5*</td>
</tr>
<tr>
<td>STZ + INS</td>
<td>42 ± 9*</td>
<td>13.2 ± 2.8</td>
<td>6.0 ± 0.6</td>
</tr>
</tbody>
</table>

Data are means ± SE (\(n = 4 – 6\)). STZ, streptozotocin; INS, insulin. *Significantly different from control (\(P < 0.05\)).

### RESULTS

Insulin treatment of diabetic rats restored blood glucose and homocysteine concentrations but was without effect on cumulative weight gain. Cumulative weight gain in diabetic rats was reduced by \(\sim 50\%\) (Table 1); insulin treatment partially prevented this difference in weight gain, but not to a level that was significantly different (\(P = 0.07\)) compared with untreated diabetic rats. In contrast, the hyperglycemia exhibited by diabetic rats (\(\sim 3\)-fold) was prevented by the administration of insulin. Total plasma homocysteine concentrations were reduced by 60% by a diabetic condition compared with the control group; however, no change in circulating homocysteine concentrations was found between control values and diabetic rats treated with insulin.

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Fig. 2. Elevated hepatic GNMT activity and protein levels in streptozotocin (STZ)-induced diabetic rats were prevented by insulin (INS) treatment (STZ + INS). At the end of the 6-day acclimation period, rats received a single intraperitoneal injection of STZ (60 mg/kg body wt) or vehicle (10 mM citrate buffer, pH 4.5). After 24 h, diabetic rats were given INS injections (1.0 U/200 \(\mu\text{L}\) saline) or the vehicle twice daily for 5 days. Hepatic GNMT activity and abundance were determined as described in MATERIALS AND METHODS. A: hepatic GNMT activity in control, diabetic, and insulin-treated diabetic rats. B: hepatic GNMT abundance in control, diabetic, and insulin-treated diabetic rats. Data are means ± SE (\(n = 4 – 6\)). *Significant difference (\(P < 0.05\)). For GNMT abundance (B), data are expressed as a percent of the control values.
GNMT induction in diabetic animals was prevented by insulin treatment. Hepatic GNMT activity was elevated 1.6-fold in STZ-induced diabetic rats compared with control values (Fig. 2A). However, treatment of diabetic rats with insulin prevented the increase in hepatic GNMT activity. Similar findings were observed with respect to the abundance of GNMT protein (Fig. 2B). GNMT abundance was 1.9-fold greater in STZ-induced diabetic rats, and the administration of insulin prevented GNMT induction.

Alterations in the hepatic activity of MS and PEMT were prevented by treatment with insulin. The hepatic activity of the folate-dependent homocysteine remethylation enzyme MS was dramatically reduced by 61% in diabetic rats, an observation that was completely prevented by insulin treatment (Table 2). Similarly, insulin treatment also prevented the 53% increase in hepatic PEMT activity exhibited by diabetic rats.

Both global and CpG island hepatic DNA hypomethylation were prevented by insulin treatment of diabetic animals. Hepatic global DNA methylation was reduced in diabetic rats compared with controls. This is demonstrated by the 1.4-fold increase in methyl acceptance by DNA measured in vitro (Fig. 3). Similarly, a diabetic state was also characterized by hepatic CpG island hypomethylation, where in vitro DNA methylation was elevated 4.7-fold in STZ-treated rats compared with control animals. For both global and CpG island methylation, insulin treatment completely prevented the altered DNA methylation exhibited in untreated diabetic rats.

Blood glucose concentrations were positively correlated with hepatic GNMT and PEMT activity and inversely correlated to plasma homocysteine concentrations. Hepatic GNMT activity was positively correlated \((r = 0.77, P < 0.001)\) with blood glucose concentrations (Fig. 4A). In contrast, plasma homocysteine concentrations were inversely correlated \((r = -0.75, P < 0.001)\) with the concentration of blood glucose (Fig. 4B). Similarly to GNMT, the hepatic activity of PEMT was positively correlated \((r = 0.69; P < 0.01)\) with blood glucose concentrations (Fig. 4C).

**DISCUSSION**

The disruption of homocysteine and methyl group metabolism has been linked to numerous pathologies; thus, a vital component in the optimization of health resides in maintaining normal methyl group and homocysteine balance as well as identifying nutritional or hormonal factors that may disrupt...
these metabolic pathways. We and others have clearly demonstrated that both type 1 and type 2 diabetes disrupts homocysteine and methyl group metabolism in humans and animals (7, 9–12, 20, 21, 23, 27, 30, 31, 34, 39–41). Specifically, type 1 diabetes results in an aberrant partitioning of methyl groups, owing to the induction of PEMT and GNMT, combined with a reduction in the activity of MS (7, 20, 21). Recently, we have also shown that type 1 diabetes was characterized by global hypomethylation of DNA (40), an epigenetic mechanism to control gene expression that has been implicated in a number of pathologies. The paramount finding in this study is that insulin administration prevents all of the aforementioned alterations in methyl group and homocysteine metabolism, including the hypomethylation of DNA. Because STZ-treated rats represent a chemically mediated model of type 1 diabetes, a significant question is whether the observed anomalies in previous reports were specific to a diabetic state or the result of chemical treatment. Our results clearly demonstrate that the disruption of methyl group and homocysteine metabolism is diabetes specific, likely owing to the lack of insulin and/or the concomitant hyperglycemia.

In addition to the prevention of DNA hypomethylation, insulin administration prevents many of the other significant changes in methyl group and homocysteine metabolism owing to diabetes. Insulin treatment has been shown to reverse the enhanced transsulfuration and folate-independent remethylation of homocysteine in both STZ-mediated diabetic rats and cultured cells treated with glucocorticoids (10, 24, 25). We have also shown in vitro that pretreatment of cells with insulin prevents the induction of GNMT by glucocorticoid administration (21) and the induction of PEMT in diabetic rats (7). Here, our finding that insulin prevents alterations in both homocysteine balance, as well as the regulation of PEMT and GNMT, is significant on a number of fronts. PEMT is considered a major consumer of methyl groups from SAM and is thus a major contributor to homocysteine production (32). GNMT is a key regulatory protein in controlling both the supply and utilization of methyl groups, thereby optimizing the methylation capacity of the cell (17, 36, 37). Hypohomocysteinemia has been noted in both diabetic humans and animals with normal renal function (10, 21, 25, 27, 38); however, as kidney function deteriorates, homocysteine disposal becomes impaired, and consequently homocysteine levels rise (9, 23, 33). Hyperhomocysteinemia is a major biomarker for a number of pathologies, including vascular disease and cancer development. Therefore, insulin administration to STZ-treated rats completely prevents alterations in both methyl group and homocysteine metabolism.

A fundamental question in this area of research is, what is the primary signal that links diabetes to the subsequent changes in methyl group metabolism? A lack of insulin, elevations in counterregulatory hormones, and/or hyperglycemia all represent logical possibilities. We have shown that glucose concentrations are highly correlated to some of the key observations noted in perturbed methyl group metabolism reported here. This suggests that circulating glucose concentrations may be a potential signal in the perturbation of methyl group metabolism in a diabetic state. However, it is also likely that a number of other compounds may be involved in regulation, and future studies will need to be directed at defining the precise mechanism. Several recent cell culture studies that were designed to mimic hyperglycemia have reported epigenetic modifications of histones that persist even after restoration of normal glucose concentrations (2, 35). It will be important to determine in future studies the specific stage of hyperglycemia that results in insulin-resistant changes in methyl group metabolism.

In summary, we have shown that disruption of methyl group metabolism by a type 1 diabetic state was prevented by insulin administration, clearly indicating that the aberrant alterations previously reported were diabetes specific. Moreover, our results provide further evidence for insulin and blood glucose as key regulators of homocysteine and methyl group metabolism and stress the importance of glycemic control in diabetics, particularly as changes in blood glucose and subsequent epigenetic marks become persistent. Importantly, similar disturbances have been reported in type 2 diabetic rat models (25) and humans with insulin resistance (28).

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DISCLOSURES

The authors have declared that no conflicts of interest exist.

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Insulin Prevents Altered Methyl Group Metabolism in Diabetes


