Modulation of Methyl Group Metabolism by Streptozotocin-induced Diabetes and All-trans-retinoic Acid

Kristin M. Nieman  
*Iowa State University*

Matthew J. Rowling  
*Iowa State University, mrowling@iastate.edu*

Timothy A. Garrow  
*University of Illinois*

Kevin Schalinske  
*Iowa State University, kschalin@iastate.edu*

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Abstract
The hepatic enzyme glycine $N$-methyltransferase (GNMT) plays a major role in the control of methyl group and homocysteine metabolism. Because disruption of these vital pathways is associated with numerous pathologies, understanding GNMT control is important for evaluating methyl group regulation. Recently, gluconeogenic conditions have been shown to modulate homocysteine metabolism and treatment with glucocorticoids and/or all-trans-retinoic acid (RA)-induced active GNMT protein, thereby leading to methyl group loss. This study was conducted to determine the effect of diabetes, alone and in combination with RA, on GNMT regulation. Diabetes and RA increased GNMT activity 87 and 148%, respectively. Moreover, the induction of GNMT activity by diabetes and RA was reflected in its abundance. Cell culture studies demonstrated that pretreatment with insulin prevented GNMT induction by both RA and dexamethasone. There was a significant decline in homocysteine concentrations in diabetic rats, owing in part to a 38% increase in the abundance of the transsulfuration enzyme cystathionine $\beta$-synthase; treatment of diabetic rats with RA prevented cystathionine $\beta$-synthase induction. A diabetic state also increased the activity of the folate-independent homocysteine remethylation enzyme betaine-homocysteine $S$-methyltransferase, whereas the activity of the folate-dependent enzyme methionine synthase was diminished 52%. In contrast, RA treatment attenuated the streptozotocin-mediated increase in betaine-homocysteine $S$-methyltransferase, whereas methionine synthase activity remained diminished. These results indicate that both a diabetic condition and RA treatment have marked effects on the metabolism of methyl groups and homocysteine, a finding that may have significant implications for diabetics and their potential sensitivity to retinoids.

Disciplines
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Modulation of Methyl Group Metabolism by Streptozotocin-induced Diabetes and All-trans-retinoic Acid*

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Kristin M. Nieman‡‡, Matthew J. Rowling‡‡, Timothy A. Garrow¶, and Kevin L. Schalinske∥

From the ‡Department of Food Science and Human Nutrition, Iowa State University, Ames, Iowa 50011 and the ¶Department of Food Science and Human Nutrition and the Division of Nutritional Sciences, University of Illinois, Urbana, Illinois 61801

The hepatic enzyme glycine N-methyltransferase (GNMT) plays a major role in the control of methyl group and homocysteine metabolism. Because disruption of these vital pathways is associated with numerous pathologies, understanding GNMT control is important for evaluating methyl group regulation. Recently, glucocorticoids and/or all-trans-retinoic acid (RA)-induced active GNMT protein, thereby leading to methyl group loss. This study was conducted to determine the effect of diabetes, alone and in combination with RA, on GNMT regulation. Diabetes and RA increased GNMT activity 87 and 148%, respectively. Moreover, the induction of GNMT activity by diabetes and RA was reflected in its abundance. Cell culture studies demonstrated that pretreatment with insulin prevented GNMT induction by both RA and dexamethasone. There was a significant decline in homocysteine concentrations in diabetic rats, owing in part to a 38% increase in the abundance of the transsulfuration enzyme cystathionine β-synthase; treatment of diabetic rats with RA prevented cystathionine β-synthase induction. A diabetic state also increased the activity of the folate-independent homocysteine remethylation enzyme betaine-homocysteine S-methyltransferase, whereas the activity of the folate-dependent enzyme methionine synthase was diminished 52%. In contrast, RA treatment attenuated the streptozotocin-mediated increase in betaine-homocysteine S-methyltransferase, whereas methionine synthase activity remained diminished. These results indicate that both a diabetic condition and RA treatment have marked effects on the metabolism of methyl groups and homocysteine, a finding that may have significant implications for diabetics and their potential sensitivity to retinoids.

Methyl group and folate-dependent one-carbon metabolism are interrelated pathways that provide for the subsequent transmethylation of various molecules using S-adenosylmethionine (AdoMet) (1). An adequate supply of methyl groups via the diet or the folate-dependent one-carbon pool and regulation of these pathways are essential in preventing associated pathologies such as cancer, vascular disease, and neural tube defects (2–4). Glycine N-methyltransferase (GNMT) (EC 2.1.1.20) is an abundant, tissue-specific protein that plays a key role in the regulation of hepatic methyl group metabolism by the enzymatic conversion of glycine and AdoMet to S-adenosylhomocysteine (AdoHcy) and sarcosine (1, 5, 6). GNMT functions to optimize the AdoMet/AdoHcy ratio, an indicator of transmethylation potential, because AdoHcy is a potent inhibitor of most AdoMet-dependent methyltransferases (5, 7). Following hydrolysis of AdoHcy, the resulting homocysteine can undergo remethylation to methionine or be irreversibly catalyzed by the transsulfuration pathway. Folate-dependent remethylation occurs with the donation of a methyl group by 5-methyltetrahydrofolate (5-CH₃-THF) through the action of B₁₂-dependent methionine synthase (MS) (8). In hepatic tissue, betaine derived from the oxidation of choline can also serve as a folate-independent source of methyl groups for homocysteine remethylation via the enzyme betaine-homocysteine S-methyltransferase (BHMT). Transsulfuration to cysteine occurs through the activity of two vitamin B₆-dependent enzymes, cystathionine β-synthase (CBS) and γ-cystathionase.

The regulation of hepatic GNMT represents an important mechanism for controlling both the folate-dependent supply of methyl groups as well as their utilization in AdoMet-dependent transmethylation reactions. GNMT activity is regulated in response to changes in methyl group status as the result of allosteric inhibition of 5,10-methylenetetrahydrofolate reductase by AdoMet (9, 10) and inhibition of GNMT by 5-CH₃-THF (11, 12), the enzymatic product of 5,10-methylenetetrahydrofolate reductase. This allows methyl groups to be conserved under conditions of deficiency by decreasing GNMT activity, whereas elevations in its activity function to dispose of excess methyl groups. In addition to allosteric control, phosphorylation of GNMT represents another posttranslational mechanism to increase the activity of GNMT and regulate methyl group metabolism (12).

Recent studies have identified both nutritional and hormonal factors that alter methyl group metabolism by targeting the key enzymes involved. All-trans-retinoic acid (RA) has been shown to induce hepatic GNMT activity and protein abundance, resulting in the loss of methyl groups for subsequent transmethylation reactions (13–16). Moreover, dexamethasone

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‡ Both authors contributed equally to this work.

§ To whom correspondence should be addressed: Dept. of Food Science and Human Nutrition, Iowa State University, 220 MacKay Hall, Ames, IA 50011. Tel.: 515-294-9230; Fax: 515-294-6193; E-mail: kschalin@iastate.edu.

∥ The abbreviations used are: AdoMet, S-adenosylmethionine; GNMT, glycine N-methyltransferase; AdoHcy, S-adenosylhomocysteine; 5-CH₃-THF, 5-methyltetrahydrofolate; MS, methionine synthase; BHMT, betaine-homocysteine S-methyltransferase; CBS, cystathionine β-synthase; RA, all-trans-retinoic acid; DEX, dexamethasone; STZ, streptozotocin; HPLC, high pressure liquid chromatography.
(DEX) was just as effective as RA in the induction of GNMT activity in rat liver and hepatoma cells, and the co-administration of both DEX and RA induced GNMT in an additive fashion (17). A diabetic state characterized by insufficient circulating concentrations of insulin and elevated levels of the counter-regulatory hormones glucagon and glucocorticoids has also been reported to alter enzymes involved in methyl group metabolism. An elevation in the activity of GNMT has been reported in alloxan-induced diabetic sheep and rats (6, 18); however, little is known about the mechanistic basis for this increase. Brosnan and co-workers have shown that under diabetic conditions, the catabolism of homocysteine was enhanced by transcriptional regulation of CBS, and these changes were prevented by treatment with insulin (19–21). Although we have shown that DEX can significantly alter methyl group metabolism and homocysteine metabolism (17), the focus in this study was to evaluate these pathways in a diabetic rat model, alone and in combination with RA, as well as to determine the effect of insulin on preventing these alterations.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**Reagents were obtained as follows: S-adenosyl-L-[methyl-3H]methionine, PerkinElmer Life Sciences; chemiluminescence Western blotting detection reagents, Amersham Biosciences; S-adenosyl-L-methionine and streptozotocin, Sigma; protease inhibitors and ylsul-L-methionine and streptozotocin, Sigma; goat anti-mouse IgG horseradish peroxidase, goat anti-rabbit IgG horseradish peroxidase, and rabbit anti-sheep IgG horseradish peroxidase, Southern Biotechnology (Birmingham, AL). GNMT and CBS antibodies were kindly provided by Yi-Ming Chen, Indiana University, Bloomington, IN (125–149 g) were housed in individual plastic cages in a room with a 12-h light/dark cycle and allowed free access to food and water. Rats were randomly assigned to treatment groups (6 rats per group) and acclimated to the control diet (17) for 5 days. Following the acclimation period, rats received a single intraperitoneal injection of either streptozotocin (STZ, 60 mg/kg body weight) in the vehicle (10 mM citrate buffer, pH 4.5) or the vehicle alone. The following day animals received a daily oral dose of RA (30 mg/kg body weight) or vehicle (corn oil) alone. Following a 5-day treatment period with RA, rats were anesthetized and whole blood samples were collected via cardiac puncture. An aliquot of whole blood was used to assess blood glucose concentrations using a commercial kit (Sigma), whereas the remaining blood was collected at 4,000 × g for 1 h at room temperature. The resulting supernatants were stored at −20 °C for subsequent analysis of homocysteine concentrations. Liver samples were removed and homogenized in 4 volumes of ice-cold phosphate-buffered saline (10 mM, pH 7.0) sucrose (0.25 M) containing 1 mM EDTA, 1 mM sodium azide, and 0.1 mM phenylmethylsulfonyl fluoride. Following centrifugation at 20,000 × g for 30 min, supernatants were stored at −70 °C with 1 mM EGTA and 1 mM EDTA to terminate the reaction, and the assay mixture was immediately applied to a Sep-Pak C18 cartridge (Waters Associates, Milford, MA) to obtain AdoMet and AdoHcy (25, 26). AdoMet and AdoHcy were separated and quantified by HPLC and fluorescence detection (29). The resulting supernatants were subjected to a two-way analysis of variance. When the analysis of variance was significant (p < 0.05), means were compared using Fish-er’s least significant difference procedure.

The following centrifugation at 18,000 × g for 8 min, supernatants were stored at −70 °C for analysis of GNMT protein abundance.

**GNMT Activity Assay—**The enzymatic activity of GNMT was determined using the method described by Cook and Wagner (24) with minor modifications, and performed in triplicate. The assay mixture (100 μl) consisted of 0.1 μl Tris buffer (pH 9.0), 1 mM glycine, 5 mM KCl, 1 mM MgCl2, 1 mM NaN3, 1 mM UDP-glucose, 1 mM β-mercaptoethanol (82.4 mM), homocysteine (100 mM), and [methyl-3H]-THF (15 mCi, 0.17 μCi/μmol) was added to liver supernatants and incubated for 1 h at 37 °C. Ice-cold water was added to terminate the reaction, and the assay mixture was immediately applied to a Sep-Pak C18 cartridge (Waters Associates, Milford, MA) to obtain AdoMet and AdoHcy.

**BHMT assay** was assayed and quantified by reverse-phase HPLC as described previously (17). A 10–20% gradient SDS-polyacrylamide gel was used to quantify the 32-kDa monomer subunit of BHMT. Subsequent separation, proteins were transferred to a nitrocellulose membrane and incubated overnight at 4 °C with a 1:4,000 dilution of the monoclonal GNMT antibody (22). The membrane was then incubated with goat anti-mouse IgG horseradish peroxidase secondary antibody for 1 h at room temperature. The membrane was developed using a similar method as described above to separate the 63-kDa GNMT subunit. A 1:2,000 dilution of the polyclonal CBS antibody was used, which was followed by incubation with a goat anti-rabbit IgG horseradish peroxidase secondary antibody. GNMT and CBS protein abundance were detected with chemiluminescence and exposed to Kodak X-Omat AR film. Densitometric analysis was performed using SigmaGel software (SPSS, Chicago, IL). For both GNMT and CBS, three samples from each treatment group were randomly chosen for analysis on a single gel for each experiment.

**Determination of Hepatic AdoMet and AdoHcy Concentrations—**Perchloric acid homogenates were centrifuged at 9,000 × g for 10 min, and the resulting supernatants were neutralized and applied to a Sep-Pak C18 cartridge (Waters Associates, Milford, MA) to obtain AdoMet and AdoHcy (25, 26). AdoMet and AdoHcy were separated and quantified by reverse-phase HPLC and UV detection (254 nm) using a mobile phase containing 30% methanol in 5 mM octane sulfonic acid (pH 4.0) operated isocratically at 1.2 ml/min.

**Analysis of BHMT and MS Activity—BHMT activity** was assayed as described previously by Garrow (27). The standard BHMT assay contained 5 mM N5-homocysteine, 2 mM betaine (0.1 μCi), and 40 μg of total protein. The activity of MS was determined as described previously (28). The assay reaction mixture containing sodium phosphate buffer (500 mM, pH 7.5), cGMP (1.3 μM), dithiothreitol (1 mM), AdoMet (10 μM), DPAPA (82.4 μM), homocysteine (100 μM), and [methyl-3H]-THF (15 mCi, 0.17 μCi/μmol) was added to liver supernatants and incubated for 1 h at 37 °C. Ice-cold water was added to terminate the reaction, and the assay mixture was immediately applied to a 1:100XL (chloride form) resin columns. Effluent fractions (3 ml of deionized water) were collected for subsequent liquid scintillation counting. For both the BHMT and MS assay, homocysteine was prepared fresh daily from a thiolactone derivative.

**Plasma Homocysteine Analysis—**Total plasma homocysteine concentrations were determined by HPLC and fluorescence detection (29). For derivatization, 10% tributyolphosphine in dimethylformamide was added to the plasma samples and subsequently incubated at 4 °C for 30 min. The reaction was terminated with ice-cold trichloroacetic acid containing 1 mM EDTA. Following centrifugation at 1,000 × g for 5 min, supernatants were added to a solution containing borate buffer (0.125 mM, pH 9.5), sodium hydroxide (1.55 mM), and 4-fluoro-7-sulfobenzo-azo (ammonium salt, 0.1%). Sodium nitroprusside (1 mM) was added to the samples prior to derivatization as an internal standard. Samples were injected onto a Waters Associates) equilibrated in a mobile phase consisting of 4% aceto- nitrite in 0.1 mM potassium phosphate buffer (pH 2.1).

**Statistical Analysis—**SigmaStat software (SPSS) was used for all statistical analyses. The mean values of each treatment group were subjected to a two-way analysis of variance. When the analysis of variance was significant (p < 0.05), means were compared using Fisher’s least significant difference procedure.
concentrations were significantly reduced and Induced CBS Abundance—Plasma homocysteine these cells.

Induction of GNMT in AR42J cells was also prevented by insulin; DEX prevented GNMT induction. Similarly, DEX-mediated induction of GNMT protein in H4IIE rat hepatoma cells (Fig. 2). Incubating DEX alone or in co-administration resulted in induction of the GNMT protein abundance (6.8-fold) to a greater extent than either treatment alone. Thus, both RA treatment and a diabetic condition modulate the expression of active GNMT in rat liver.

Both Diabetes and RA Treatment Induced Active GNMT—STZ-treated rats exhibited a 1.9-fold increase in hepatic GNMT activity compared with the control values (Fig. 1A). RA increased GNMT activity 2.5-fold compared with control rats and was significantly greater than the degree of induction in STZ-treated rats. Hepatic GNMT activity was highest in the rats receiving both STZ and RA (2.6-fold), although this increase was not significantly different from RA treatment alone. The changes in GNMT activity in diabetic and RA-treated rats were also reflected in the abundance of the protein, where a 5.3- and 4.8-fold increase was observed, respectively (Fig. 1B). Moreover, co-administration of STZ and RA significantly induced GNMT protein abundance (6.8-fold) to a greater extent than either treatment alone. Thus, both RA treatment and a diabetic condition modulate the expression of active GNMT in rat liver.

Induction of GNMT by RA and/or DEX was Prevented by Insulin Treatment—As reported previously (17), both RA and DEX alone or in co-administration resulted in induction of the GNMT protein in H4IIE rat hepatoma cells (Fig. 2). Incubating cells with insulin for 24 h prior to treatment with RA and/or DEX prevented GNMT induction. Similarly, DEX-mediated induction of GNMT in AR42J cells was also prevented by insulin; however, RA was not an effective signal to induce GNMT in these cells.

STZ Treatment Reduced Plasma Homocysteine Concentrations and Induced CBS Abundance—Plasma homocysteine concentrations were significantly reduced (~48%) in both groups of STZ-treated rats regardless of RA administration (Fig. 3). Although we have found previously that RA alone was effective at lowering circulating homocysteine levels (14), the 28% decrease observed under these experimental conditions did not reach statistical significance. The hypohomocysteinemia exhibited by diabetic rats appears to be due, in part, to a 38% increase in the abundance of CBS, the initial enzyme in the irreversible catabolism of homocysteine (Fig. 4). However, CBS activity is regulated allosterically, and thus flux studies conducted in vivo would be required to evaluate the transsulfuration pathway. Interestingly, RA treatment of diabetic rats prevented CBS induction (Fig. 4), even though plasma homocysteine concentrations remained diminished (Fig. 3).

STZ and RA Treatments Differentially Stimulate Homocysteine Remethylation Enzymes—In addition to transsulfuration, the reduced plasma homocysteine levels also appear to reflect changes in the activity of enzymes involved in homocysteine remethylation. BHMT activity was significantly increased 3.3-fold in diabetic rats, whereas the activity of MS was reduced 52% (Table II). RA did not significantly alter either enzyme, although there was a trend (p = 0.061) for increased MS activity, similar to our previous observations (14). RA was effective at reducing the activity of BHMT in diabetic rats, but did not prevent the STZ-mediated decrease in MS activity.

STZ-mediated Elevations in the Hepatic Concentrations of AdoMet and AdoHcy Were Prevented by RA Treatment—A diabetic condition resulted in a significant increase in the hepatic concentrations of AdoMet (169%) and AdoHcy (90%) (Table III). Although RA treatment did not significantly alter AdoMet and

| Weight gain | Blood glucose |
|-------------|---------------|
| g | mg/dl |
| Control | 77 ± 2* | 147 ± 17* |
| STZ | 18 ± 3* | 539 ± 19* |
| RA | 73 ± 1* | 165 ± 17* |
| STZ + RA | 26 ± 5* | 435 ± 51* |

**Fig. 1.** Induction of hepatic GNMT activity and abundance in STZ-mediated diabetic and RA-treated rats. Diabetic (single dose of STZ, 60 mg/kg body weight) rats were treated daily with RA (30 μmol/kg body weight) for 5 days. Liver samples were removed and the activity and abundance of GNMT was determined as described under “Experimental Procedures.” A, GNMT enzyme activity in diabetic and non-diabetic rats following administration of RA or corn oil. Data are expressed as means ± S.E. (n = 6), and bars denoted with different letters are significantly different (p < 0.05). B, GNMT protein abundance in diabetic and non-diabetic rats following administration of RA or corn oil. A monoclonal GNMT antibody (22) was used for Western blot analysis, and a representative immunoblot is shown. Data are expressed as means ± S.E. (n = 3), and bars denoted with different letters are significantly different (p < 0.05).
AdoHcy concentrations in non-diabetic rats, it did prevent their accumulation in diabetic animals. Because AdoMet and AdoHcy are allosteric regulators of a number of enzymes involved in folate, methyl group, and homocysteine metabolism, it is difficult to determine the collective metabolic effect of these changes in diabetic rats in the absence of flux measurements using isotopic tracers.

**DISCUSSION**

Methyl groups, homocysteine, and the folate-dependent one-carbon pool represent interrelated metabolic processes that are important in health and disease. Because GNMT is a vital regulatory protein that functions to control the supply and utilization of methyl groups, it is paramount to understand how nutritional and/or hormonal factors modulate its expression. Our earlier work demonstrated that RA and DEX were independent signals to induce active GNMT in rat liver and in cultured hepatoma cells (17). Here, we have extended these findings by demonstrating that a diabetic condition, mediated by treatment with STZ, also has a profound impact on the activity of hepatic GNMT. Previous rat studies using alloxan-induced diabetes reported similar increases (85%) in GNMT activity (6), whereas treatment with glucagon were less pronounced (23%) (20); however, the mechanistic basis for this increase in GNMT activity has not been thoroughly explored. A novel aspect of our studies demonstrates that for both diabetes and RA, the abundance of GNMT protein was markedly elevated, indicating that GNMT was regulated by transcriptional and/or translational mechanisms or possibly at the level of degradation of the protein. It is not known if regulation of GNMT expression in a diabetic state is the result of an increase in glucocorticoid levels or a lack of insulin. The cell culture studies shown here clearly demonstrate that pretreatment with insulin prevented GNMT induction by both DEX and RA. It will be important in future studies to more fully characterize this diabetic condition with alterations in methyl group metabolism. Interestingly, the responsiveness of GNMT expression to DEX was a more universal signal than RA. It is not known if regulation of GNMT protein abundance is controlled by mRNA levels or protein stability, or possibly at the level of translation. It is also not known if regulation involves specific recognition sites for insulin and RA, or if similar recognition sites are involved. This is a novel aspect of our studies demonstrating that for both diabetes and RA, the abundance of GNMT protein was markedly elevated, indicating that GNMT was regulated by transcriptional and/or translational mechanisms or possibly at the level of degradation of the protein. It is not known if regulation of GNMT expression in a diabetic state is the result of an increase in glucocorticoid levels or a lack of insulin. The cell culture studies shown here clearly demonstrate that pretreatment with insulin prevented GNMT induction by both DEX and RA. It will be important in future studies to more fully characterize this diabetic condition with alterations in methyl group metabolism. Interestingly, the responsiveness of GNMT expression to DEX appears to be cell type specific. We have found in other studies that DEX was a more universal signal than RA for GNMT induction across a number of tissues and cell lines, including those that do not express detectable GNMT, such as...
the human hepatoma cell line HepG2.\textsuperscript{2} It is not clear how RA, DEX, and/or a diabetic state alter the expression of GNMT. Previous studies on the regulation of GNMT activity have found it to be at the posttranslational level by phosphorylation and allosteric inhibition by 5-CH\textsubscript{3}-THF (11, 12, 30, 31). Based on the known actions of glucocorticoids and RA with respect to regulation of gene expression, it is likely that the induction of GNMT is the result of increased transcription. In support of this hypothesis, we have found that pretreatment of hepatoma cells with actinomycin D prevented GNMT induction by DEX and RA, and abundance of the protein appears to reflect changes in its synthesis rate.\textsuperscript{3} However, GNMT has not been reported to contain a retinoic acid- nor a glucocorticoid-response element in its promoter region (32). Therefore, it is likely that additional intracellular signals are required to directly mediate regulation of GNMT expression. Nonetheless, we cannot exclude the possibility that increases in phosphorylation of the protein have a role in increasing GNMT activity, particularly as glucagon and glucocorticoids can exert their action via alterations in protein kinase activity.

Homocysteine concentrations reflect the collective balance between production from AdoMet-dependent transmethylation reactions, remethylation to methionine, and catabolism via the transsulfuration pathway. As has been reported (19, 20), we also found that a diabetic state was characterized by a reduction in circulating homocysteine levels. This change in homocysteine homeostasis appears to reflect an increase in the activity of BHMT and MS, although we have found in previous studies that RA does increase MS when provided for a longer period of time (14). However, it should be noted that the activity and abundance of GNMT remained elevated in rats treated with RA. This further illustrates the need for metabolic flux measurements under in vivo conditions to determine the outcome of these interactions.

The implications of these results for humans are profound, as diabetes and its complications are a significant health problem and the use of pharmacological retinoid compounds has risen dramatically in recent years (35). In addition to folate, optimal metabolism of methyl groups and homocysteine is dependent on a number of B-vitamins including B\textsubscript{12}, B\textsubscript{6}, and riboflavin, as well as an adequate source of methyl groups such as methionine and choline. Moreover, it is well known that the human population has a significant prevalence of polymorphic enzymes important in methyl group, homocysteine, and folate-dependent one-carbon metabolism (36). Taken together, these nutritional, genetic, and hormonal factors underscore the need for understanding the relationship between these metabolic pathways and diabetes. As most type I diabetics control their disease by the use of insulin, there are nonetheless likely many individuals who do not adequately monitor their condition. Moreover, many of the changes noted in our study were recently demonstrated in a type II diabetes (Zucker diabetic fatty rat) model, including an increase in hepatic CBS, BHMT, and AdoMet (37).

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\textsuperscript{9} 2 M. J. Rowling, K. M. Nieman, and K. L. Schalinske, unpublished data.

\textsuperscript{10} 3 M. J. Rowling, K. M. Nieman, and K. L. Schalinske, unpublished data.

\textsuperscript{11} 4 S. E. Knoblock, E. B. Nonnecke, M. J. Rowling, and K. L. Schalinske, unpublished data.
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