Effects of diabetes and insulin on betaine-homocysteine S-methyltransferase expression in rat liver

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Ratnam, Shobhitha, Enoka P. Wijekoon, Beatrice Hall, Timothy A. Garrow, Margaret E. Brosnan, and John T. Brosnan. Effects of diabetes and insulin on betaine-homocysteine S-methyltransferase expression in rat liver. Am J Physiol Endocrinol Metab 290: E933–E939, 2006. First published December 13, 2005; doi:10.1152/ajpendo.00498.2005.—Elevation of plasma homocysteine reflects a balance between its synthesis via dysfunction have decreased levels. (15). Plasma homocysteine; diabetes; insulin; glucocorticoids; remethylation pathways. Betaine-homocysteine methyltransferase (BHMT, EC 2.1.1.5) is one of the enzymes involved in the remethylation pathway. BHMT, a major zinc metalloenzyme in the liver, catalyzes the transfer of methyl groups from betaine to homocysteine to form dimethylglycine and methionine. We have previously shown that plasma homocysteine levels and the transsulfuration pathway are affected by diabetes. In the present study, we found increased BHMT activity and mRNA levels in livers from streptozotocin-diabetic rats. In the rat hepatoma cell line (H4IIE cells), glucocorticoids (triamcinolone) increased the level and rate of BHMT mRNA synthesis. In the same cell line, insulin decreased the abundance of BHMT mRNA and the rate of de novo mRNA transcription of the gene. Thus the decreased plasma homocysteine in various models of diabetes could be due to increased homocysteine removal brought about by a combination of increased transsulfuration of homocysteine to cysteine and increased remethylation of homocysteine to methionine by BHMT.

homocysteine; diabetes; insulin; glucocorticoids; remethylation

AN ELEVATION OF PLASMA HOMOCYSTEINE is recognized as an independent risk factor for the development of Alzheimer’s disease, premature arteriosclerosis, thrombosis, and connective tissue disorders, including skeletal abnormalities, osteoporosis, and fractures (3, 19, 20, 22, 38, 41). Cardiovascular disease is a major complication of diabetes. Plasma homocysteine reflects a balance between its synthesis via dysfunction have decreased levels. (15). Plasma homocysteine; diabetes; insulin; glucocorticoids; remethylation pathways. Betaine-homocysteine methyltransferase (BHMT, EC 2.1.1.5) is one of the enzymes involved in the remethylation pathway. BHMT, a major zinc metalloenzyme in the liver, catalyzes the transfer of methyl groups from betaine to homocysteine to form dimethylglycine and methionine. We have previously shown that plasma homocysteine levels and the transsulfuration pathway are affected by diabetes. In the present study, we found increased BHMT activity and mRNA levels in livers from streptozotocin-diabetic rats. In the rat hepatoma cell line (H4IIE cells), glucocorticoids (triamcinolone) increased the level and rate of BHMT mRNA synthesis. In the same cell line, insulin decreased the abundance of BHMT mRNA and the rate of de novo mRNA transcription of the gene. Thus the decreased plasma homocysteine in various models of diabetes could be due to increased homocysteine removal brought about by a combination of increased transsulfuration of homocysteine to cysteine and increased remethylation of homocysteine to methionine by BHMT.

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lente human insulin was from Eli Lilly (Indianapolis, IN). Radiolabeled [methyl-14C]betaine and [methyl-14C]choline chloride were purchased from American Radiochemical (St. Louis, MO). Dowex 50W-X4 ion exchange resin was from Bio-Rad (Hercules, CA). H4IIE (rat hepatoma) cells were obtained from American Type Culture Collection (Manassas, VA). Dulbecco’s modified Eagle’s medium (DMEM), trypsin-EDTA, fetal bovine serum (FBS), penicillin, streptomycin, and other tissue culture supplies were purchased from Invitrogen Canada (Burlington, ON, Canada). Triaminocline and insulin were from Sigma Chemicals (Oakville, ON, Canada). RNA later was from Ambion (Austin, TX). One-step RT-PCR kit was purchased from Qiagen (Mississauga, ON, Canada). BHMT primers were custom made by Invitrogen Canada. Rat BF-2.2 amplimer set was purchased from CloneTech (Palo Alto, CA). Bacitracinonic acid (BCA) protein assay kit was from Pierce Chemical (Rockford, IL). All other reagents used were obtained commercially and were of the highest purity available.

**Animals and in vivo procedures.** All procedures were approved by Memorial University’s Institutional Animal Care Committee and are in accordance with the Canadian Council on Animal Care. Male Sprague-Dawley rats weighing 280 – 350 g were used in all studies. They were weight matched at the beginning of the study. The animals were fed laboratory chow ad libitum and had free access to water. Diabetes was induced by a single intravenous injection of 100 mg/kg streptozotocin (dissolved in 10 mM citrate buffer, pH 4.5). Diabetic rats were treated with Novolin ultralente human insulin subcutaneously for 5 days, at which point insulin administration was stopped for one-half of the rats. The other half received insulin for another 5 days. Insulin was administered at 0900 and 1900; the dose was adjusted to maintain blood glucose close to normal values as measured with an Ames Glucometer II, using a drop of blood obtained by tail prick. On the day of the study, animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/kg), and blood samples were taken from the abdominal aorta. Heparinized tubes containing the blood samples were held on ice until plasma was separated by centrifugation at 3,700 g. Blood samples were held on ice until plasma was separated by centrifugation at 3,700 g. The supernatants were analyzed by HPLC using a Yvidac C18 column (model 2187PS4) that was equilibrated with 96% of buffer A (50 mM NaH2PO4 containing 10 mM heptanesulfonic acid at pH 3.2) and 4% acetonitrile. SAM and Sadenosyl-l-homocysteine (SAH) were separated by using a gradient of 96–80% of buffer A and 4–20% of acetonitrile for 15 min. SAM and SAH peaks were detected at 258 nm and were quantitated using Millennium software (Waters, Milford, MA).

**Reverse transcriptase-polymerase chain reaction.** Total RNA was isolated from H4IIE cells as described (26). Total RNA was prepared from freeze-clamped livers by a rapid guanidinium thiocyanate method. Total RNA (2 μg) was reverse transcribed using a one-step reverse transcription kit and was amplified. A 1,219-bp fragment (nucleotides +5 to +1224 relative to the start codon) of BHMT was amplified using the following primers: 5’-ACGGATCCGGCGCAAGAA-3’ and 5’-CCTGTCGCGATGTTGATTTT-3’. A 764-bp fragment of rat β-actin was amplified using amplimer set primers. PCR products were separated on 1% agarose. Ethidium bromide-stained bands were visualized by UV illumination. The blots were scanned with the ChemiImager 4000 and quantified with AlphaEase software (Alpha Innotech, San Leandro, CA).

**PCR-based nuclear run-on analysis.** H4IIE cells grown in monolayer were either untreated or treated for 6 h with 1 μM triaminocline with or without insulin (1 μM), harvested, and washed three times by centrifugation at 400 g with ice-cold Dulbecco’s phosphate-buffered saline (DPBS). Isolation of the nuclei and a PCR-based nuclear run-on assay were performed essentially as described (28). Briefly, cells (5 x 10^6) were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 1 mM KCl containing 0.5% NP-40) and incubated on ice for 5 min. They were then layered over 30% sucrose in lysis buffer without NP-40 and centrifuged at 600 g for 5 min. Nuclear pellets were resuspended in glycerol storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl₂, 0.1 mM EDTA), flash frozen in liquid nitrogen, and stored at −70°C until use. Nuclear suspensions (200 μl) were split into two aliquots and incubated for 30 min at 30°C in 20% glycerol, 30 mM Tris-HCl (pH 8.0), 2.5 mM MgCl₂, 150 mM KCl, 1 mM EDTA, 1 mM DTT, and 40 μg/mL Proteinase (Promega, Madison, WI). One aliquot contained 5 mM each of rCTP, rATP, and rGTP and rUTP. rNTPs were added to the second aliquot. After the addition of yeast tRNA (20 μg), nuclear RNA was extracted by the acid-phenol/phenol-chloroform method, and RT-PCR was performed as described above. The relative transcription rate at the time of nuclear isolation was assessed by the difference in the amplicons generated from transcripts obtained in the presence or absence of exogenous rNTPs. Data were normalized to the transcription rate of the β-actin gene.

**Statistical analysis.** All data were analyzed by GraphPad Prism software. Data are presented as means ± SD of six individual experiments unless otherwise stated. Statistical comparisons were done using one-way analysis of variance followed by a Newman-Keuls multiple comparison test. A P value of <0.05 was taken to indicate a significant difference.
RESULTS

Effect of diabetes on BHMT and CDH. Both BHMT (Fig. 1A) and CDH activities (Fig. 1B) were increased by 50% in streptozotocin-induced diabetic rats and returned to normal upon insulin treatment. To determine whether the observed decrease in BHMT enzyme activity was regulated at the level of transcription, BHMT mRNA was measured in treated and untreated diabetic rat livers. BHMT mRNA levels were higher in diabetic rat livers compared with control rat livers, and this trend was reversed by insulin treatment. When normalized for β-actin mRNA, diabetic rats showed a 60% increase in the level of BHMT mRNA (Fig. 2).

Effect of diabetes on hepatic concentrations of SAM and SAH. Hepatic concentrations of SAM and SAH were significantly elevated in streptozotocin-induced diabetic rats; SAM concentrations were 25% above control values and SAH concentrations were increased by 122%. Although insulin treatment reduced the levels of both SAM and SAH, their levels remained higher than normal. The SAM/SAH ratio decreased in diabetes. Insulin treatment resulted in an increase in the SAM/SAH ratio. However, it remained lower than control values (Table 1).

Effect of triamcinolone and insulin on H4IIE cells. To examine whether the changes in BHMT seen in diabetic rats could be attributed to a direct response to insulin and/or its counterregulatory hormones, BHMT enzyme activity was assayed in H4IIE cells, a rat hepatoma cell line that retains many of the characteristics of primary hepatocytes. BHMT activity was significantly higher in H4IIE cells stimulated with triamcinolone (a glucocorticoid analog). This increase was reversed by insulin (Fig. 3A). Steady-state mRNA was measured by RT-PCR analysis to determine whether the activation of BHMT was related to an increase in transcription. H4IIE cells constitutively expressed low but detectable levels of BHMT mRNA (Fig. 3B), which increased two- to threefold when cells were treated with triamcinolone (1 μM) for 18 h. This stimulation of mRNA expression by glucocorticoids was significantly inhibited when insulin (1 μM) was added. The level of BHMT induction by glucocorticoids and the inhibition of glucocorticoid-mediated BHMT expression by insulin were dose dependent. Although a concentration of 10 nM insulin reduced glucocorticoid-induced BHMT steady-state mRNA levels by ~40%, a concentration of 100 nM insulin brought these levels down to ~25% of the maximal stimulation (Fig. 4).

Effect of triamcinolone and insulin on BHMT transcription rate. Changes in the BHMT transcription rate in response to glucocorticoids and insulin were determined by means of PCR-based nuclear run-on experiments. The effects of these hormones on the BHMT transcription rate paralleled the effects on BHMT mRNA levels (Fig. 5). Triamcinolone increased the BHMT transcription rate, and insulin abolished this effect.

Table 1. Hepatic SAM and SAH in control, diabetic, and insulin-treated diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
<th>Insulin-Treated Diabetic</th>
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<tr>
<td>SAM, nmol/g</td>
<td>152.7 (9.4)</td>
<td>187.6 (11.9)</td>
<td>173.4 (7.4)</td>
</tr>
<tr>
<td>SAH, nmol/g</td>
<td>12.3 (2.0)</td>
<td>27.2 (4.8)</td>
<td>19.1 (4.3)</td>
</tr>
<tr>
<td>SAM/SAH ratio</td>
<td>12.8 (2.5)</td>
<td>7.0 (1.0)</td>
<td>9.4 (1.7)</td>
</tr>
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Results are expressed as means (SD) for 6 animals. SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine. Differences in superscript letters represent significant differences within rows, P < 0.05.
Therefore, insulin plays a direct role in the regulation of BHMT transcription.

**DISCUSSION**

Type 1 diabetic patients with nephropathy have increased homocysteine levels. In contrast, diabetic patients with normal creatinine levels (indicating normal renal function) have lower than normal plasma homocysteine levels (27). Recently, it has been shown that insulin resistance is associated with reduced plasma homocysteine levels (30). In our study, untreated streptozotocin-treated rats were clearly diabetic. The plasma glucose levels in these rats were threefold higher than control levels (27.8 ± 2.1 vs. 9.1 ± 1.7). Insulin treatment returned these values to normal levels (7.8 ± 9.8). Plasma homocysteine levels were decreased threefold in diabetic rats (3.2 ± 0.5 vs. 9.1 ± 1.7). Insulin treatment increased the plasma homocysteine levels but they remained higher than normal levels (6.1 ± 2.2). This is in agreement with our previous report (16). Our previous experiments showed that plasma creatinine levels are normal in the streptozotocin-diabetic model, indicating that changes in the glomerular filtration rate were not responsible for the observed lowering of plasma homocysteine levels (16). We have also previously shown that the hepatic catabolism of homocysteine via the transsulfuration pathway is activated in diabetic rats with a concomitant lowering of homocysteine (16, 26). The present study shows that the decrease in plasma homocysteine levels is also associated with an increase in the activity of BHMT, a key contributor to remethylation. Our data clearly show that insulin treatment of the diabetic rats reversed this increase. The elevation of BHMT enzyme activity was accompanied by an increase in the steady-state levels of BHMT mRNA, which were also decreased by insulin treatment, suggesting that circulating insulin levels play a role in the regulation of folate-independent homocysteine remethylation, possibly at the level of BHMT transcription. Recent studies by Neiman et al. (24) also showed that hepatic BHMT activity was increased in streptozotocin-treated rats. Although an earlier study from our laboratory showed no change in BHMT activity in streptozotocin-treated diabetic rats (16), we have confirmed the findings of Neiman et al. (24) by using new methodology to measure the enzyme activity. Use of less sensitive methods may have contributed to this discrepancy. Our data show that CDH, which catalyzes the first of two successive oxidation steps in the biosynthetic conversion of choline to betaine, is also elevated in diabetic rats. To our...
knowledge, this is the first report to show that hepatic CDH activity is increased in diabetic rats and that insulin treatment normalizes it.

There is now considerable evidence that glucocorticoids and insulin play important roles in regulating the expression of genes encoding regulatory enzymes of homocysteine metabolism. The expression of liver-specific glycine N-methyltransferase is regulated by glucocorticoids (31). Our previous work has shown that these hormones can regulate CBS expression (26). We have now demonstrated that the action of glucocorticoids on BHMT gene expression is direct and specific by using a rat hepatoma cell line. This cell line has been used extensively to study glucocorticoid-mediated induction of enzymes involved in various metabolic pathways including that of phosphoenolpyruvate carboxykinase and tyrosine aminotransferase (33, 34). Stimulation of these cells by glucocorticoids induced a significant increase in the BHMT mRNA levels, which was suppressed by insulin. To our knowledge, this is the first report that shows insulin to have a direct role in the regulation of folate-independent remethylation of homocysteine. Our studies have indicated that the regulation of BHMT occurs at the level of transcription. The actual mechanism by which these hormones regulate this enzyme is not known. The rat BHMT promoter region is not yet mapped. The 5'-flanking region (considered to be the promoter region) of the mouse BHMT has been elucidated. However, no consensus sequence for direct insulin or glucocorticoid response is seen in this sequence (23).

Why might BHMT activity be regulated by diabetes? One possibility is that this may relate to the effect of hormones on SAM levels. Our results show that both SAM and SAH are elevated in diabetic rats and that insulin treatment reduced their levels. SAM is a critical intracellular regulator of methionine metabolism. It is an allosteric activator of CBS and a potent inhibitor of MTFHR, which converts N\(^5,10\)-methylenetetrahydrofolate to N\(^5\)-methylytetrahydrofolate (6). Hepatic SAM levels can be modulated by the major hormones of fuel metabolism. They are elevated in streptozotocin-diabetic (24), Zucker diabetic fatty (type 2) (46), and hyperglucagonemic rats (17). Glucocorticoids are also known to elevate SAM synthetase activity and mRNA abundance (9). Because elevated levels of hepatic SAM tend to activate transsulfuration and (via its inhibitory effect on MTFHR) decrease remethylation of homocysteine by methionine synthase, it is possible that folate-independent remethylation via BHMT activity would need to be enhanced. Our results showed that the SAM/SAH ratio was lower in diabetic animals and that insulin treatment returned it to normal levels. The marked increase in SAH may be particularly important since this is a potent inhibitor of many methyltransferases (6). SAH is removed by SAH hydrolase, which converts it to homocysteine and adenosine. This enzyme is reversible and thought to be close to equilibrium in vivo. It is known that agents that impair the removal of adenosine can result in increased SAH levels. For example, an inhibitor of adenosine deaminase (2'-deoxycoformycin) used to treat T cell acute lymphoblastic leukemia increases lymphoblast SAH levels 20-fold, causing a marked decrease in the SAM/SAH ratio (12). It has been shown that adenosine accumulates in livers of streptozotocin-diabetic rats due to decreased activity of adenosine kinase, whose expression is normally stimulated by insulin (32). We speculate, therefore, that the increased hepatic SAH levels may be due to these increased adenosine levels. The possible importance of these increased SAH levels (e.g., in affecting hepatic DNA methylation) remains an important topic for future investigation.

Changes in BHMT activity may also relate to phospholipid metabolism. BHMT is thought to be functionally linked to phosphatidylethanolamine methyltransferase (PEMT), which converts phosphatidylethanolamine (PE) to phosphatidylcholine (PC), by three successive SAM-dependent methylation reactions. Although most BHMT is cytosolic and PEMT is found in the endoplasmic reticulum, a fraction of both of these enzymes is localized to the bile canalicular membrane. This is thought to facilitate the synthesis of PC at close proximity to the biliary secretion site(s) and emphasizes the role of BHMT in bile production (37). The biliary secretion of PC is very large. It has been estimated that in normal livers the daily biliary output from the human liver is about 5000 mg (24). The biliary secretion of PC is closely related to the phosphatidylcholine turnover in the liver and is frequently elevated in various liver diseases (24). The biliary secretion of PC is very large. It has been estimated that in normal livers the daily biliary output from the human liver is about 5000 mg (24). The biliary secretion of PC is closely related to the phosphatidylcholine turnover in the liver and is frequently elevated in various liver diseases (24).
In conclusion, the direct and specific effects of glucocorticoids on BHMT induction and its repression by insulin demonstrated in this work provide new insights into the mechanism by which insulin regulates the metabolism of homocysteine, a highly atherogenic amino acid. The specific mechanism by which insulin mediates its inhibitory effect on BHMT transcription remains to be determined.

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GRANTS

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