Effects of Insulin and Glucose on Cellular Metabolic Fluxes in Homocysteine Transsulfuration, Remethylation, *S*-Adenosylmethionine Synthesis, and Global Deoxyribonucleic Acid Methylation

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Background: The mechanisms underlying the impact of pathophysiological elevations in insulin or glucose on hepatic cellular homocysteine kinetics is not fully understood.

Objective: The objective of the study was to investigate the impact of elevated insulin/glucose on hepatic homocysteine kinetics at the cellular level.

Design and Methods: Effects of insulin and glucose on homocysteine remethylation and transsulfuration metabolic fluxes were investigated in a cell model using stable isotopic tracers and gas chromatography/mass spectrometry. The methylation status was assessed by *S*-adenosylmethionine (adoMet), the adoMet to *S*-adenosylhomocysteine ratio, DNA methyltransferase activity, and methylated cytidine content of DNA. The expression profile of homocysteine remethylation, transmethylation, and transsulfuration-associated genes was determined.

Results: Insulin increased cellular homocysteine production primarily by its inhibition of transsulfuration. When cells were exposed to elevated insulin and glucose, homocysteine remethylation was enhanced, which consequently increased intracellular adoMet concentrations by inducing adoMet synthase activity. Elevated glucose further enhanced DNA methyltransferase activity that subsequently led to increased global DNA methylation.

Conclusions: We demonstrated the novel finding of a direct promoting effect of high cellular insulin or glucose exposure on homocysteine remethylation, adoMet synthase activity, and adoMet synthesis. We also provided new evidence indicating that when hepatic tissue is exposed to elevated insulin or glucose, the cellular methylation balance can be altered, which may have potential epigenetic impacts gene regulation in diabetic individuals. These findings in a cell line may or may not reflect what happens in humans. *In vivo* studies on the homocysteine transmethylation fluxes and DNA methylation in diabetic state are underway. **(*J Clin Endocrinol Metab* 94: 1017–1025, 2009)**

Diabetes mellitus results from defects in insulin secretion and/or insulin action. It currently affects approximately 4% of the population worldwide (1). Diabetes, commonly accompanying hyperglycemia, hyperlipidemia, hypertension, obesity, and abnormal homocysteine metabolism (2), is associated with a marked increase in the occurrence (3) and mortality (4) of cardiovascular diseases. As an independent risk factor, hyperhomocystinemia may contribute to the increased risk of cardiovascular diseases in diabetes (5, 6).

We recently showed that glycine *N*-methyltransferase knockout mice exhibited high hepatic *S*-adenosylmethionine (adoMet) levels and had hypoglycemia, suggesting an association between perturbed adoMet-dependent transmethylation and abnormal glucose metabolism (7). The present study was undertaken to

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2008-2038 Received September 16, 2008. Accepted December 10, 2008.
First Published Online December 16, 2008
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Abbreviations: adoHcy, *S*-adenosyl homocysteine; adoMet, *S*-adenosyl methionine; BHMT, betaine-homocysteine *S*-methyltransferase; CβS, cystathionine β-synthase; CTH, cystathionase; DNMT, DNA methyltransferase; MAT, *S*-adenosyl methionine synthase; MdC, 5-methyldeoxycytidine; MS, methionine synthase; MTHFR, methylene-tetrahydrofolate reductase.

investigate the impact of glucose on hepatic intracellular transmethylation and homocysteine kinetics.

Moderately elevated homocysteine levels have been associated with macrovascular disease in patients with or without diabetes (8, 9). Perturbations in homocysteine metabolism have been reported in diabetes: patients with either type 1 or type 2 diabetes having renal dysfunction exhibit elevated plasma homocysteine levels, whereas type 1 diabetic patients without clinical signs of renal dysfunction have lower homocysteine level (10). Patients with type 1 diabetes have lower rates of homocysteine-methionine remethylation but increased transsulfuration (11), and insulin treatment normalizes transsulfuration and remethylation in these patients (11). Hyperinsulinemia induced by a hyperinsulinemic-euglycemic clamp stimulates homocysteine transmethylation and transsulfuration in the plasma of healthy humans (12). Such insulin-induced increments of methionine transmethylation, homocysteine transsulfuration, and clearance were found impaired in type 2 diabetic subjects (13). Postmethionine load hyperhomocystinemia is present in patients with non-insulin-dependent diabetes mellitus (14), suggesting impaired transsulfuration in this condition.

Alterations in homocysteine metabolic enzymes have been reported in various diabetic states. In rats fed a high-fat sucrose diet, plasma insulin levels correlated positively with homocysteine and with methylene-tetrahydrofolate reductase (MTHFR) activity, and inversely correlated with cystathionine-β-synthase (CβS) activity (15). In experimentally induced insulin-dependent diabetes, CβS and cystathionine-γ-lyase activity is elevated in rat liver, and such elevation is normalized by insulin treatment (16, 17). In a streptozotocin-induced diabetes rat model, injection of insulin decreased the activity of transsulfuration enzyme activities, hence increased plasma homocysteine in a dose-dependent manner (18). Zucker diabetic rats with elevated insulin have increased hepatic CβS and betaine-homocysteine *S*-methyltransferase (BHMT) (19). Jacobs *et al.* (16) found hepatic BHMT, MTHFR, and methionine synthase (MS) activity unchanged in the streptozotocin model (16). Nieman *et al.* (20) reported that streptozotocin treated rats lacking insulin have increased BHMT and reduced MS. Increases in the insulin concentration leads to significant decrease MTHFR activities in hepatocytes (21). CβS-1b promoter activity is decreased by insulin treatment in HepG2 cells (17).

Liver is the major insulin-responsive organ in charge of systemic glucose homeostasis under normal conditions. Hepatic glucose production is tightly regulated by insulin (22, 23) and increased hepatic glucose production is associated with hyperglycemia in diabetes (23). The physiological function and impact on cellular single-carbon metabolism vary among different cell types and depend on the expression of tissue-specific metabolic enzymes. Most mammalian remethylation through the BHMT pathway is confined to the liver and kidneys (24), whereas remethylation of homocysteine catalyzed by MS occurs in most tissues (25). Transsulfuration occurs only in the liver, kidneys, small intestines, pancreas, and brain (26).

The mechanisms underlying the impact of such pathophysiological elevations in insulin or glucose on hepatic cellular homocysteine kinetics is not fully understood. The present study was undertaken to investigate the specific actions of insulin and glucose on hepatic cellular homocysteine metabolism. We performed *in vitro* kinetic experiments to focus on individual metabolic pathways that could be altered by elevated insulin or glucose. Results from the present study help to elucidate the regulation of homocysteine metabolism in the diabetic state at the cellular level.

Materials and Methods

Chemicals and cell culture

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise specified. HepG2 human hepatocellular carcinoma cell line (ATCC) was grown in α-MEM containing 10% bovine calf serum, 0.12% $NaHCO_3$, penicillin, streptomycin, amphotericin, and 5% CO_2 at 37C. To investigate specifically how insulin and glucose may influence hepatic homocysteine metabolism at the cellular level, comparable concentrations of insulin or glucose seen in insulin-resistant diabetic patients were used in the culture model. In each experiment, cells were cultured in either low methionine (10 μM) or sufficient methionine (100 μM) media, treated with either PBS (control) or 300 mg/dl glucose or 200 U/ml insulin for 72 h. The glucose concentration of the control media was 100 mg/dl. The 3-d culture conditions were intended to produce a chronic, rather than acute, effect of insulin/glucose on homocysteine metabolism.

Determination of homocysteine production

In mammalian cells, cellular concentrations of homocysteine and cysteine are maintained at low levels by regulated production and efficient removal of these thiols (27). Therefore, homocysteine concentration in medium reflected the quantity of produced and exported homocysteine by these cells during the experimental period, which was measured as previously described (28).

Determination of adoMet and *S*-adenosyl homocysteine (adoHcy)

After the incubation period, cells were harvested, washed, and pelleted. Pellets were hand homogenized in 0.4 M ice-cold perchloric acid and centrifuged. Supernatants were stored at −80 C until analyzed. Intracellular adoMet and adoHcy were determined by the previously described procedure (29). Values were normalized to cellular protein content that was determined using the bicinchoninic assay method.

Determination of *S*-adenosylmethionine synthase (MAT) enzyme activity

After the treatment period, cells were harvested, washed, pelleted, and homogenized in ice-cold buffer consisting of 0.154 M KCl/50 mM Tris-HCl and EDTA (pH 7.4). The MAT activity was determined by quantifying the adoMet production by 300 μg cellular protein. Reaction mixtures consisted of 80 mM Tris-HCl/50 mM KCl (pH 7.4), 5 mM ATP, 40 mM $MgCl_2$, and cellular protein of enzyme solution. After preincubation, methionine (0.1 mM) was added to initiate the enzyme reaction. The incubation carried at 37 C for 30 min and was terminated by ice-cold perchloric acid. In this assay, adoMet level of supernatant fraction without reaction represents baseline value. The MAT activity was calculated as the adoMet production after subtracting baseline adoMet and expressed as nanomoles adoMet formedmg $protein^{-1}$ $30min^{-1}$.

Determination of DNA methyltransferase (DNMT) activity

DNMT activity was measured by incubating cell lysates containing10 μg of protein with 0.5 μg of poly[d(I-C)d(I-C)] template (Amer-

sham Pharmacia Biotech, Sunnyvale, CA) and 3 μCi [^{3}H]adoMet (NEN Life Science Products, Boston, MA) for 2 h at 37 C as described previously (30). The reaction was terminated, and DNA template was purified by organic extraction and ethanol precipitation. Pellets were resuspended in 0.3 M NaOH, incubated at 37 C for 1 h, spotted onto GF/C filter papers (Whatman Inc., Florham Park, NJ), and processed for liquid scintillation counting.

Determination of global DNA methylation

After incubation period, cells were harvested and DNA was isolated from cells using a standard phenol/chloroform/isoamyl alcohol procedure (31). The degree of global DNA methylation was determined as measured content of 5-methyldeoxycytidine (MdC) in the DNA described previously (32). The molar percent of MdC, *i.e.*100 × MdC/(dC+MdC), was calculated.

Isotope tracer studies

The impacts of insulin or glucose on homocysteine metabolic pathways were investigated in separate kinetic experiments. To investigate the flux in overall homocysteine remethylation, methionine in medium was replaced with $^{13}C_5$-methionine (Cambridge Isotope Laboratories, Woburn, MA). When $^{13}C_5$-methionine enters the methionine cycle, it will lose one labeled carbon by adoMet-dependent methyltransferase reactions and generates $^{13}C_4$-homocysteine. As $^{13}C_4$-homocysteine remethylates to methionine by receiving one unlabeled carbon from 5-CH_3-tetrahydrofolate or betaine, it turns into $^{13}C_4$-methionine, and the ratio between existing remethylated methionine (methionine M+4) and unmetabolized methionine tracer (methionine M+5) species reflect the degree of overall remethylation of homocysteine. The folate-dependent homocysteine remethylation by the carbon of serine is calculated from the enrichments of the serine (serine M+1) and methionine (methionine M+1). In this experiment, media were supplemented with L-[5,5,5-$^2H_3$]leucine (199.5 μM) so we could estimate protein turnover. After the labeling period, medium was removed and cells washed twice with ice-cold PBS. Isotopic enrichment was determined in electron capture negative ionization mode by gas chromatography/mass spectrometry as described previously (33).

Expression profile of homocysteine metabolism genes

Total RNA was isolated and integrity was checked by electrophoresis. RNA was converted to cDNA by RT-PCR. Gene expression was determined by quantitative real-time PCR ABI7000 (Applied Biosystem Inc., Foster City, CA). The expression of each gene was calculated by normalizing the threshold cycle value of target gene to that of the control housekeeping gene (18sRNA). Abbreviations of gene symbols are shown in the legend of Fig. 1.

Results

Effects of insulin and glucose on cellular homocysteine production

We separately examined the impact of glucose and insulin and found that high glucose alone did not affect homocysteine production, whereas insulin significantly increased homocysteine production in HepG2 cells (Table 1). We postulated that the elevated homocysteine induced by insulin we observed in HepG2 cells was due to inhibition of transsulfuration that was examined by the labeling experiments discussed below.

Insulin inhibited homocysteine transsulfuration pathways

Neither insulin nor glucose affected cytoplasmic serine or leucine enrichments under conditions with sufficient methionine when using ^{13}C-serine and d_3-leucine as tracers (Table 1). In addition, cellular proteins enriched in d_3-labeled leucine did not differ among control (40.8 ± 0.1%), insulin-treated (39.2 ± 1.8%, $P > 0.05$ *vs.* controls), or glucose-treated (37.2 ± 1.5%, $P > 0.05$) cells, indicating that tracer uptake and protein turnover remained unaltered in these experimental conditions.

Insulin treatment reduced mRNA expressions of transsulfuration genes CβS and cystathionase (CTH) (Fig. 1A), increased homocysteine, and decreased cysteine excretion (Table 1). Furthermore, the proportion of enriched cystathionine (labeled by ^{13}C-serine) decreased by 23% when cells were treated with insulin, and the degree of these reductions was comparable with the relative increase in homocysteine concentrations (23%) (Table 1). Under methionine restriction, approximate 7.5% of cystathionine enrichment came from ^{13}C-serine. When these methionine-depleted cells were treated with insulin, no cystathionine enrichment was detected, indicating that transsulfuration was completely inhibited by insulin under this condition. These results demonstrated that insulin inhibited hepatic homocysteine transsulfuration regardless of methionine status, and we concluded that insulin induced homocysteine accumulation in HepG2 cells by inhibiting transsulfuration.

High cellular glucose did not alter homocysteine transsulfuration flux

It was reported previously that glucose treatment at 300 mg/dl for 72 h increased CβS up to 8-fold in HepG2 cells (21). However, we did not find evidence of elevated homocysteine transsulfuration in glucose-treated cells. First, high cellular glucose did not affect homocysteine or cysteine excretion in HepG2 cells. Second, high glucose did not alter homocysteine transsulfuration flux (79.4 ± 0.03% in control *vs.* 78.9 ± 0.9% in glucose treated, $P = 0.49$) (Table 1), nor did glucose induce the transsulfuration gene CβS or CTH (Fig. 1B). Because we found that high glucose alone does not reduce homocysteine directly, the inverse correlation between plasma glucose and homocysteine levels observed in type 2 diabetic humans (34) could be attributed to an indirect mechanism.

Insulin and glucose enhanced methionine synthesis by promoting homocysteine remethylation when methionine is in demand

In mammalian cells, homocysteine can undergo remethylation for methionine synthesis via the folate-dependent MS pathway or the folate-independent BHMT pathway (23), or it can be catabolized to cysteine via the transsulfuration pathway. In the present study we discovered that high glucose significantly increased homocysteine remethylation. When methionine was restricted, both insulin and glucose increased the overall homocysteine remethylation flux (Table 2). During methionine repletion, insulin modestly but significantly increased folate-dependent homocysteine remethylation flux without affecting overall homocysteine remethylation (Table 3). Under methionine restriction, insulin substantially increased folate-dependent remethylation by about 19% and increased overall remethylation flux by about 8% (Table 3). These results suggested that

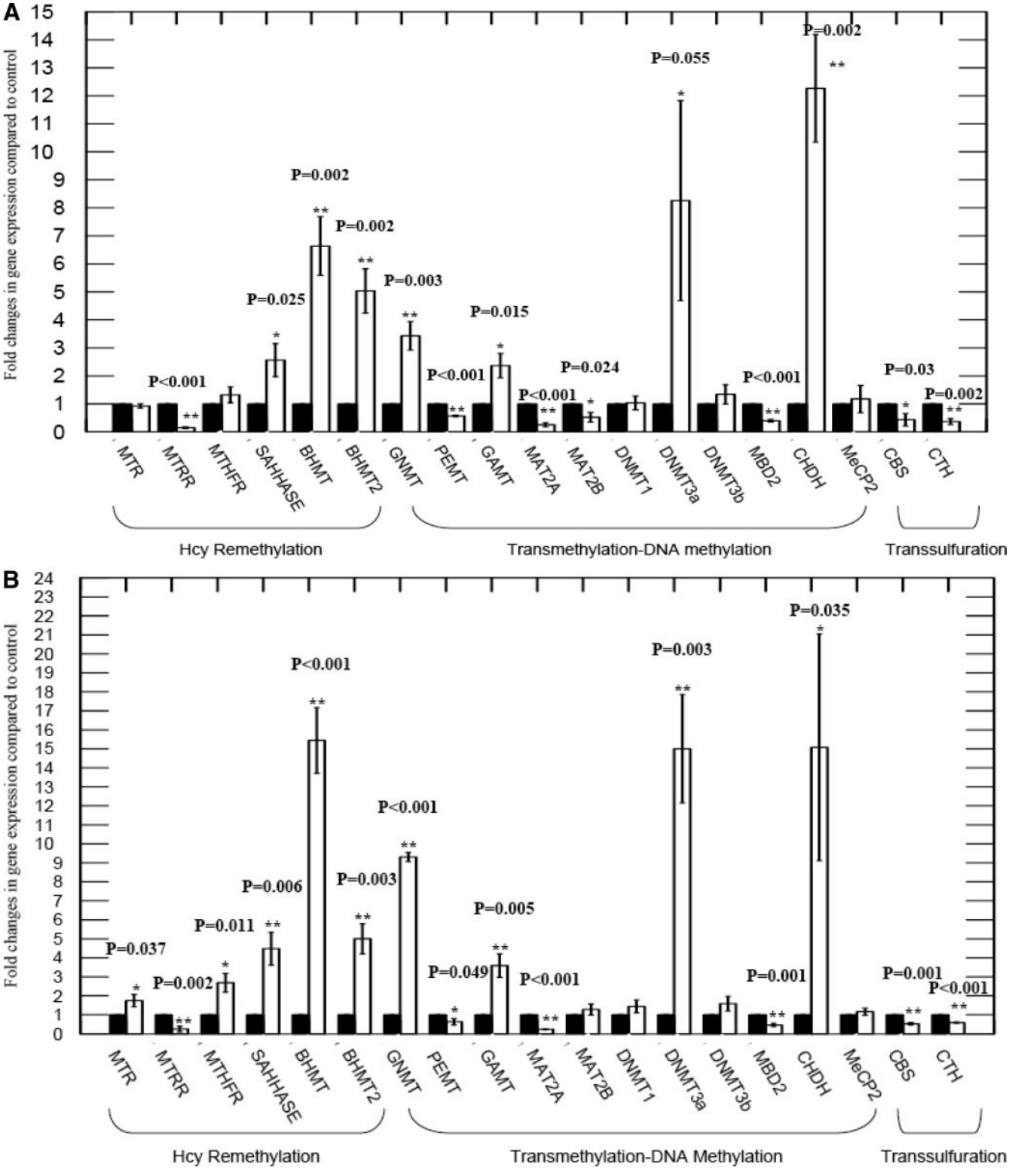

FIG. 1. Effects of insulin on the mRNA expression of remethylation, transmethylation, and transsulfuration-associated genes in HepG2 cells. Data are presented as mean ± SD (n = 3). Values with *different symbols* are significantly different. *, $P < 0.05$; **, $P < 0.01$. MTR, Methionine synthase; MTRR methionine synthase reductase; SAHH, *S*-adenosylhomocysteine hydrolase; GNMT, glycine-*N*-methyltransferase; PEMT, phosphatidylethanolamine *N*-methyltransferase; GAMT, guanidinoacetate methyltransferase; MBD2, methyl-CpG binding domain; CHDH, choline dehydrogenase; MeCP2, methyl-CpG binding domain protein 2. A, *Closed* and *open bars* represent control and insulin, respectively. B, *Closed* and *open bars* represent control cells and glucose (300 mg/dl for 72 h)-treated cells, respectively.

TABLE 1. Impacts of insulin and glucose on homocysteine/cysteine production and cytoplasmic homocysteine transsulfuration in HepG2 cells

	Leucine+3 enrichment	Serine+1 enrichment	Cystathionine+1 enrichment	Relative cystathionine+1 from ser+1	Homocysteine (nmol/10 cells)	Cysteine (μmole/10 cells)
Control	0.463 ± 0.007	0.252 ± 0.037	0.198 ± 0.026[a,b]	79.4 ± 0.03%[c]	3.30 ± 0.19[e]	3.71 ± 0.09[g]
Insulin	0.465 ± 0.001	0.272 ± 0.006	0.151 ± 0.006[a]	56.4 ± 1.0%[d]	4.13 ± 0.18[f]	2.68 ± 0.09[h]
Change, %				−23.0 ± 1.0%	22.9 ± 7.8%	−27.8 ± 4.1%
P value	0.691	0.537	0.133	**0.001**	**0.008**	**<0.001**
Glucose	0.466 ± 0.013	0.276 ± 0.006	0.213 ± 0.003[b]	78.9 ± 0.9%[c]	3.41 ± 0.10[e]	3.26 ± 0.05[i]
Change, %				−0.5 ± 0.9	1.7 ± 6.5	−12.0 ± 3.0
P value	0.778	0.471	0.482	0.486	0.726	**0.002**

All data are presented as means ± SD (n = 3). Values with *different letters* and values in *bold* are significantly different ($P < 0.05$). Control cells were cultured in α-MEM medium. Cells in the insulin group were treated with 1 μM insulin for 72 hrs. Cells in the glucose group were treated with 300 mg/dL glucose for 72 hrs. Percent change and *P* value were analyzed by comparing to controls. Medium are supplemented with L-[^{13}C]serine (237.8 μM, 50% of total serine) combined with L-[5,5,5-$^{2}H_3$]leucine (399 μM, 50% of total leucine).

insulin may preferentially induce the folate-dependent pathway in this model. Furthermore, folate-dependent homocysteine remethylation was significantly increased by insulin and by glucose (Table 3), especially when methionine was restricted (Table 3). With respect to the homocysteine remethylation genes, insulin induced BHMT1 and BHMT2 by 7- and 5-fold, respectively (Fig. 1A). Glucose treatment increased BHMT1 and BHMT2 by more than 15- and 5-fold, respectively (Fig. 1B). Induction of these genes may contribute to the increased remethylation and transmethylation induced by glucose exposure. Taken together, both insulin and glucose could enhance methionine synthesis by promoting homocysteine remethylation when methionine is in demand. We further investigated whether such alterations affected MAT activity and intracellular adoMet stores.

Effects of insulin and glucose on MAT activity, adoMet, adoHcy, DNMT activity, and global DNA methylation

MAT activity was induced by insulin ($P = 0.05$, compared with controls) and glucose ($P = 0.009$, compared with controls) (Table 4), yet the MAT2A gene was not induced in either insulin- (Fig. 1A) or glucose-treated cells (Fig. 1B), suggesting that the induction of MAT was posttranscriptional. Methionine restriction decreased intracellular adoMet and increased adoHcy, presumably resulting from a low methionine supply for adoMet synthesis and an accelerated conversion of adoMet to adoHcy for the demand of methylation reactions. Insulin treatment mildly (~4%) increased adoMet in the presence of adequate methionine and increased adoMet by about 17% under methionine restriction (Table 4).

TABLE 2. Effects of insulin and glucose on cytoplasmic homocysteine remethylation pathways under methionine-sufficient and methionine-restricted conditions

	Met+4 enrichment	Met+5 enrichment	Overall homocysteine remethylation
Methionine sufficient			
Control[a]	0.054 ± 0.004	0.356 ± 0.036	2.5 ± 0.4
Insulin[b]	0.057 ± 0.001	0.379 ± 0.013	2.4 ± 0.7%
Change, %			−0.05 ± 0.85
P	0.333	0.424	0.878
Glucose[c]	0.064 ± 0.006	0.325 ± 0.026	6.8 ± 0.3%
Change, %			4.4 ± 0.42
P	0.221	0.486	**<0.001**
Methionine restricted			
Low met control[d]	0.105 ± 0.013	0.233 ± 0.021	23.3 ± 1.6%
Low met +insulin[b]	0.139 ± 0.013	0.236 ± 0.036	31.4 ± 1.6%
Change *vs.* control, %	32.0 ± 12.33	1.29 ± 15.52	8.0 ± 1.4%
P	0.124	0.928	**0.032**
Low met +glucose[c]	0.169 ± 0.007	0.273 ± 0.013	32.6 ± 0.2%
Change *vs.* control, %	60.57 ± 6.38	17.44 ± 5.42	8.5 ± 0.7%
P	**0.026**	0.146	**0.006**

Data are presented as means ± SD (n = 3). Values with *different letters* and values in *bold* are significantly different ($P < 0.05$). Met, Methionine.

[a] HepG2 cells were cultured in α-MEM.

[b] With 1 μM insulin treatment for 72 h.

[c] With 300 mg/dl glucose treatment for 72 h.

[d] Medium methionine concentration was 10 μM in low methionine experiments.

TABLE 3. Effects of insulin and glucose on cytoplasmic folate-dependent remethylation under methionine-sufficient and -restricted conditions

	Leu+3 enrichment	Ser+1 enrichment	Met+1 enrichment	Folate-dependent remethylation
Methionine sufficient				
Control[a]	0.463 ± 0.007	0.252 ± 0.038	0.044 ± 0.006	17.3 ± 0.1
Insulin[b]	0.465 ± 0.001	0.272 ± 0.006	0.058 ± 0.004	21.4 ± 1.1
Change, %				4.1 ± 1.4
P	0.691	0.537	0.11	**0.032**
Glucose[c]	0.466 ± 0.013	0.276 ± 0.006	0.055 ± 0.001	19.9 ± 0.001
Change, %				2.4 ± 0.19
P	0.778	0.471	0.129	**0.001**
	Ser+1 Enrichment	**Met+1 enrichment**	**Leu+3 enrichment**	**Folate-dependent remethylation**
Methionine restricted				
Low met control[d]	0.151 ± 0.011	0.028 ± 0.001	0.272 ± 0.014	19.3 ± 2.8%
Low met+insulin[b]	0.157 ± 0.031	0.062 ± 0.004	0.264 ± 0.032	38.7 ± 3.3%
Change *vs.* control, %	4.78 ± 16.1	119.8 ± 15.3	−2.02 ± 9.62	19.0 ± 4.2%
P	0.728	**0.009**	0.807	**0.013**
Low met+glucose[c]	0.037 ± 0.036	0.015 ± 0.001	0.257 ± 0.043	40.1 ± 0.5%
Change *vs.* control, %	6.93 ± 0.93	−46.43 ± 5.1	−6.07 ± 6.50	21.5 ± 0.7%
P	0.201	**0.012**	0.369	**<0.001**

Values in *bold* are significantly different ($P < 0.05$). Met, Methionine.

[a] HepG2 cells were cultured in α-MEM.

[b] With 1 μM insulin treatment for 72 h.

[c] With 300 mg/dl glucose treatment for 72 h.

[d] Medium methionine concentration was 10 μM in low methionine experiments.

Glucose treatment increased adoMet by 17.6% during methionine repletion and increased adoMet by 35.2% during methionine depletion. Glucose increased adoHcy by 9.5% when methionine was adequate and drastically increased adoHcy content by 79.7% under methionine restriction (Table 4). Because the tissue concentrations of these substrates are of particular importance for regulating methionine cycle and transmethylation, we then examined whether such an altered adoMet to adoHcy ratio subsequently impacted DNMT activity and DNA methylation. Genes involved in transmethylation were also investigated. Insulin induced guanidinoacetate methyltransferase mRNA expression by more than 2-fold and increased choline dehydrogenase by more than 12-fold, implying that insulin could alter some methylation reactions via transcriptional regulation. However, we found that insulin did not alter DNMT activity or methylcytidine in HepG2 cells (Table 4). On the other hand, glucose increased the mRNA expressions of guanidinoacetate methyltransferase, choline dehydrogenase, and DNMT3a (Fig. 1B). In addition to the transcriptional induction of DNMT3a, glucose also significantly increased DNMT activity by more than 40% when methionine was adequate (untreated *vs.* treated cells, $P = 0.025$) and increased DNMT activity by 63% when methionine was restricted ($P < 0.001$). Finally, glucose significantly increased the content of MdC in DNA (Table 4). Among these experimental conditions, the degree of global DNA methylation positively correlated with intracellular adoMet concentrations ($r = 0.880$, $P = 0.011$) and tended to inversely correlate with adoHcy concentrations ($r = 0.781$, $P = 0.078$). These data implied that insulin secretion could help maintain adoMet levels when methionine was in demand due to increased MAT activity.

TABLE 4. Effects of insulin and glucose on intracellular content adoMet, adoHcy, adoMet to adoHcy ratio, MAT activity, DNA methyltransferase activity, and 5-methyldeoxycytidine contents of genomic DNA

	adoMet	adoHcy	adoMet to adoHcy ratio	MAT activity	DNMT activity	MdC (%)
Control	451.0 ± 4.9^{a}	44.2 ± 1.1^{f}	10.2 ± 0.2^{j}	4.24 ± 0.44^{o}	37000 ± 6557^{r}	2.78 ± 0.05^{v}
Insulin	469.0 ± 3.0^{b}	43.5 ± 0.4^{f}	10.8 ± 0.1^{k}	5.24 ± 0.44^{p}	39000 ± 6230^{r}	2.80 ± 0.09^{v}
Glucose	552.1 ± 7.6^{c}	48.4 ± 1.7^{g}	11.4 ± 0.3^{l}	6.68 ± 0.68^{q}	52333 ± 3786^{s}	3.04 ± 0.06^{w}
Low met control	34.4 ± 1.7^{d}	51.7 ± 2.1^{g}	0.7 ± 0.03^{m}		34946 ± 783^{t}	2.04 ± 0.11^{x,y}
Low met insulin	40.2 ± 1.4^{e}	79.4 ± 2.0^{h}	0.5 ± 0.01^{n}		37820 ± 1046^{r}	1.92 ± 0.05^{x,z}
Low met glucose	46.5 ± 4.0^{e}	92.9 ± 2.3^{i}	0.5 ± 0.04^{n}		56920 ± 1792^{u}	2.20 ± 0.06^{y}

Data are presented as means ± SD (n = 3). Values with *different letters* are significantly different ($P < 0.05$). Met, Methionie. Control cells were cultured in α-MEM medium. Cells in the insulin group were treated with 1 μM insulin for 72 hrs. Cells in the glucose group were treated with 300 mg/dL glucose for 72 hrs. In low met group, medium contained 10 μM in low methionine experiments. adoMet and adoHcy data are expressed as the unit of (pMole/mg protein). adoMet/adoHcy: *S*-adenosyl methionine to *S*-adenosyl homocysteine ratio. MAT activity data are expressed as the unit of (nmol adoMet formed * mg protein^{-1} * 30 min^{-1}) DNMT activity data are expressed as the unit of (cpm/mg protein). dmC (%): percentage of 5-methyldeoxycytidine in genomic DNA.

Our results also indicated that elevated cellular glucose levels may alter global DNA methylation by enhancing DNMT activity in liver.

Discussion

The methionine cycle in mammalian cells synthesizes adoMet, uses it for transmethylation, hydrolyzes adoHcy, and remethylates homocysteine. The impact of hormonal balance on the regulation of one-carbon metabolism is not fully illustrated (35). The diabetic state caused by insulin deficiency/resistance may have distinct impacts on homocysteine catabolism and its reuse among various tissues because those involved enzymes have tissue-specific distributions and regulation by insulin and glucose. Using HepG2 cells as a model system to investigate cellular homocysteine kinetics in hepatic tissue, we demonstrated the novel finding of a direct promoting effect of insulin and high glucose exposure on homocysteine remethylation, MAT activity, and adoMet synthesis. High glucose further increased DNMT activity and affected global DNA methylation in this model. Our kinetic experiments helped better elucidate the abnormal hepatic homocysteine metabolism in the diabetic state at the cellular level.

Insulin increases cellular homocysteine production primarily by inhibition of transsulfuration, and insulin increases homocysteine remethylation flux when methionine is restricted

We demonstrated in HepG2 cells that insulin significantly increased homocysteine production by reducing transsulfuration via transcriptional inhibition of CβS and CTH.

Using this cell model to specifically investigate the direct effects of insulin, we found that insulin preferentially induced folate-dependent homocysteine remethylation pathways when methionine was restricted in this model. If insulin plays a crucial role for hepatic homocysteine remethylation, hepatic use of homocysteine for methionine synthesis might be impaired or less efficient in pathological conditions with insulin deficiency. We suggest that normal secretion and function of insulin is probably required for maintaining MS activity in liver, and BHMT and/or CβS could be the major pathways for hepatic homocysteine use and catabolism in individuals lacking insulin. We recognize that this study was conducted neither in human subjects nor from cells obtained from normal human subjects directly, and future *in vivo* studies are warranted.

The impacts of insulin and glucose on specific homocysteine metabolic pathways in HepG2 cells are shown in Fig. 2, A and B, respectively.

The action of glucose on homocysteine transsulfuration at the cellular level

Homocysteine was found to inversely correlate with blood glucose levels in diabetic patients (34), suggesting a potential regulatory mechanism of glucose on homocysteine homeostasis *in vivo*. MTHFR activity decreased whereas CβS activity increased as glucose concentrations increased in HepG2 cells (21). Based on these data, one would predict that high glucose may have enhanced homocysteine clearance mainly via an elevation in transsulfuration. In contrast, we found that high glucose did not increase transsulfuration, nor did it increase cysteine production in these cells. Conversely, high glucose resulted in reduced cysteine excretion from HepG2 cells. Adults with trisomy 21 not taking vitamin B supplements had a normal homocysteine concentration compared with controls (36),

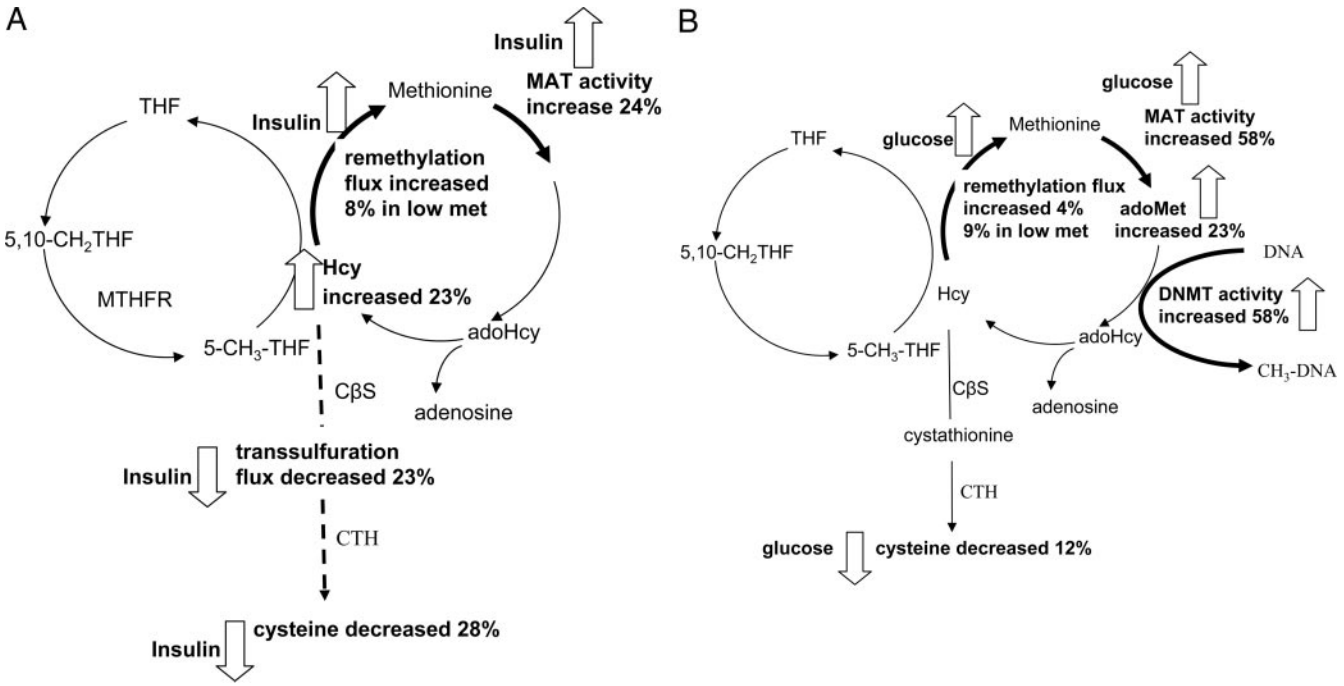

FIG. 2. A proposed model for cellular homocysteine kinetics when liver exposed to (A) insulin and (B) glucose. 5-CH_3THF, 5-Methyltetrahydrofolate; 5,10-CH_2THF, 5, 10-methylene tetrahydrofolate; Hcy, homocysteine.

suggesting that elevated CβS did not necessarily increase homocysteine catabolism. We then examined the postulation that high glucose decreases cellular homocysteine by promoting remethylation.

High glucose increases overall remethylation flux but it increases folate-dependent remethylation only when methionine is restricted

During hyperglycemia, cellular glucose greatly increases in many tissues. In the present study, we demonstrated that elevated glucose enhanced homocysteine remethylation flux in HepG2 cells, thereby suggesting that the high glucose may not be directly responsible for MS inhibition in streptozotocin-treated rats (20). Our data also suggested that glucose induced BHMT gene expression by more than 15-fold and modestly induced BHMT-dependent remethylation flux. It was reported that MTHFR activity decreased when the medium glucose concentrations increased from 100 to 300 mg/dl in the HepG2 cells (21). It is possible that such inhibitory effect of high glucose accounts for the rather modest change in folate-dependent homocysteine remethylation, by limiting methylated folate in these cells. When methionine was restricted, glucose enhanced cytoplasmic folate-dependent flux by about 22% and overall homocysteine remethylation flux by about 9%, suggesting that glucose could preferentially induced MS-dependent remethylation during methionine restriction. Numerous genes involved in homocysteine remethylation including methionine synthase, MTHFR, *S*-adenosylhomocysteine hydrolase, BHMT1, and BHMT were significantly induced by glucose, and we suggest that high cellular glucose may promote methionine synthesis, regardless of methionine status. Our kinetic experiments indicated that high glucose increases homocysteine remethylation in HepG2 cells; hence, elevated blood glucose may reduce homocysteine levels by increasing remethylation.

If high glucose enhances hepatic homocysteine remethylation flux *in vivo*, our findings could provide an explanation, at least in part, for the inverse correlation between homocysteine and blood glucose levels observed in diabetic patients (34) and help delineate the metabolic route of homocysteine in liver under pathophysiological conditions.

High glucose increases MAT activity, methylation potential, DNMT activity, and global DNA methylation

We demonstrated that methionine restriction decreased intracellular adoMet and increased adoHcy, presumably due to the increased conversion of adoMet to adoHcy to meet the demand for multiple methylation reactions. Methionine restriction also resulted in a reduction in MAT and DNMT activities and decreased the content of MdC. Our *in vitro* labeling studies suggested that insulin and glucose could increase cellular adoMet levels by enhancing homocysteine remethylation and inducing MAT activity. High glucose further increased DNMT activity and MdC content under such experimental conditions. Under sufficient methionine conditions, insulin modestly increased the methylation potential and MAT activity but the DNMT activity and MdC content appeared unchanged. On the other hand, glucose increased the methylation potential (adoMet to adoHcy ratio), MAT activity, DNMT activity, and global DNA methylation. Methylation potential was drastically reduced under methionine restriction and MdC. Global DNA methylation was positively correlated with intracellular adoMet concentrations and tended to inversely correlate with adoHcy concentrations. To our knowledge, this is the first study demonstrating that the altered levels of adoMet and adoHcy induced by high glucose further impacts DNA methylation by induction of DNMT activity.

We hope the present study help delineate the metabolic route of homocysteine in the liver under pathophysiological conditions, such as uncontrolled elevated cellular glucose. These results provide new evidence indicating that when hepatic tissue is exposed to insulin or glucose, the cellular methylation balance could be altered. The alteration of DNA methylation by increased glucose may have potential epigenetic impacts on gene regulation in diabetic individuals. These findings in a cell line may or may not reflect what happens *in vivo*, and human studies to confirm these findings are needed. *In vivo* studies on the effects of abnormally elevated glucose on transmethylation fluxes and DNA methylation are underway.

The present study gives us new insights into the regulation of insulin and glucose on cellular homocysteine metabolism. Understanding the regulatory mechanisms by which insulin and glucose affect homocysteine metabolism may lead to a better management of the secondary complications accompanying diabetes.

Acknowledgments

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This work was supported by Grants NSC95-2320-B-005-008-MY3 (E.-P.I.C.) and DOH 96-TD-D-113-039 (E.-P.I.C.).

Disclosure Statement: The authors have nothing to disclose.

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