Effects of a High-Fat–Sucrose Diet on Enzymes in Homocysteine Metabolism in the Rat

Vivian Fonseca, Aliza Dicker-Brown, Subramanian Ranganathan, Wei Song, R. James Barnard, Louis Fink, and Philip A. Kern

Hyperhomocysteinemia (HH) and hyperinsulinemia are both risk factors for cardiovascular disease. To examine the effects of hyperinsulinemia on homocysteine metabolism, we fed rats a high-fat–sucrose (HFS) diet and then measured the hepatic mRNA and activity of 2 key enzymes involved in this metabolic pathway: 5,10-methylenetetrahydrofolate reductase (MTHFR) and cystathionine-β-synthase (CβS). Fischer rats made insulin-resistant by a HFS diet were examined at 6 months and 2 years of age and compared with control rats fed a low-fat, complex-carbohydrate (LFCC) diet. At the end of 6 months, the HFS rats were heavier than the LFCC rats (214 ± 3.4 *v* 188 ± 1.4 g, $P < .01$). There were no differences in blood glucose between HFS and LFCC rats; however, plasma insulin and homocysteine concentrations were elevated in HFS rats (insulin, 56 ± 12 *v* 14.5 ± 2.9 μU/mL; homocysteine, 10.77 ± 0.9 *v* 6.89 ± 0.34 μmol/L, $P < .01$). Hepatic CβS enzyme activity was significantly lower in HFS compared with LFCC rats (0.45 *v* 0.64 U/mg, $P = .0001$), and this decrease was reflected in a decrease of the CβS mRNA concentration. In contrast, hepatic MTHFR enzyme activity and mRNA concentration were significantly elevated in the HFS group compared with controls (HFS and LFCC, 8.62 and 4.8 nmol/h/mg protein, respectively, $P = .0001$). These changes in plasma homocysteine, CβS, and MTHFR were significantly correlated with the degree of obesity and hyperinsulinemia. Fasting plasma insulin correlated significantly and positively with plasma homocysteine ($r = .51$, $P < .01$) and MTHFR activity ($r = .48$, $P < .01$) and negatively with CβS activity ($r = -.54$, $P < .001$). CβS and MTHFR activities were inversely correlated with each other ($r = -.58$, $P < .001$). In conclusion, rats fed a HFS diet are hyperinsulinemic, and the hyperinsulinemia is associated with an elevated homocysteine concentration and changes in 2 key enzymes in homocysteine metabolism.

HOMOCYSTEINE is a thiol-containing amino acid metabolized by remethylation to methionine or transsulfuration to cysteine.[1,2] Hyperhomocysteinemia (HH), with elevations in plasma total homocysteine (tHcy) both in the fasting state and following a methionine load, has been recently recognized as an important risk factor for cardiovascular disease.[2,3] The most dramatic examples of HH and macrovascular disease occur in subjects with homocystinuria, which is caused by mutations in one of several enzymes involved in the metabolism of methionine. Although homocystinuria is rare, moderate elevations of plasma homocysteine are common and represent an important risk factor for the development of premature cardiovascular disease.[3]

Patients with type 2 diabetes have accelerated coronary artery disease, much of which can be explained by traditional risk factors such as dyslipidemia. However, patients with type 2 diabetes are usually insulin-resistant, and previous studies have demonstrated that insulin resistance and hyperinsulinemia per se are risk factors for atherosclerosis.[4,5] We recently examined plasma tHcy concentrations in patients with type 2 and type 1 diabetes. We found that patients with type 2 diabetes more frequently had HH, especially subjects with accelerated macrovascular disease,[6] whereas patients with type 1 diabetes did not have HH.

Because of these observations in type 2 diabetic patients and because insulin affects the metabolism of several amino acids, we wondered whether insulin resistance or hyperinsulinemia would affect plasma tHcy concentrations. In addition, we wished to study 2 enzymes, methylenetetrahydrofolate reductase (MTHFR) and cystathionine-β-synthase (CβS), which are known to be important in the control of tHcy metabolism. Although several other enzymes are involved in the metabolism of tHcy, we chose to study these 2 because abnormalities of these enzymes have been most frequently implicated in causing HH in humans. To address these questions, we chose to study plasma tHcy and the hepatic expression of MTHFR and CβS in rats fed a high-fat–sucrose (HFS) diet, which is known to make rats hyperinsulinemic and insulin-resistant.[7-12] As described herein, the hyperinsulinemic rats demonstrated HH, along with reciprocal changes in CβS and MTHFR.

MATERIALS AND METHODS

Animal Model

Diets. We examined the effects of 2 different long-term diets on homocysteine metabolism. Female Fischer rats at 8 weeks of age were assigned to either a low-fat, complex-carbohydrate (LFCC) or HFS diet group, as described by us previously.[6] The percentage of calories as protein, fat, and carbohydrate was 21%, 6%, and 73%, respectively, for the LFCC diet and 21%, 39.5%, and 39.5%, for the HFS diet. The diets were prepared in powder form by Purina Test Diets (Purina, St Louis, MO) with vitamin-free casein as the source of protein. The source of fat in the HFS diet consisted of 90% lard and 10% corn oil. Both diets contained a standard mineral and vitamin mix. Food and water were available ad libitum on a 12-hour light/dark cycle. There was no difference in 24-hour urine creatinine excretion (7.9 ± 1.0 *v* 10.6 ± 6.5 mg/24 h) in the LFCC and HFS groups, respectively.

From the Department of Medicine, Division of Endocrinology, and Department of Pathology, University of Arkansas for Medical Sciences, Little Rock Central Arkansas Veterans Healthcare System, Little Rock, AR; and Department of Physiological Science, University of California at Los Angeles, Los Angeles, CA.

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Address reprint requests to Vivian Fonseca, MD, Department of Medicine, Section of Endocrinology SL53, Tulane University School of Medicine, 1430 Tulane Ave, New Orleans, LA 70112.

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Both groups were maintained on their respective diets and were killed at either 6 months or 2 years of age. There were 16 rats in each group, with 8 each killed at 6 months and at 2 years. The rats were weighed before death, and blood insulin was measured as previously described.[7] Briefly, the rats were fasted overnight and anesthetized with chloralhydrate (300 mg/kg intraperitoneally) and blood was obtained via cardiac puncture. The sample was centrifuged for 20 minutes, and the plasma was frozen at −70°C. In previous studies, HFS rats have been shown to be nondiabetic and insulin-resistant, with a high fasting insulin concentration and normal fasting glucose.[8] Furthermore, this rat model has been shown in previous studies to manifest other features of the "insulin resistance syndrome," including obesity, hypertension, hypertriglyceridemia, and enhanced clotting.[7]

Plasma homocysteine. The plasma homocysteine level was measured using a modification of the method described by Ubbink et al.[13] The plasma was thawed and treated with tri-*n*-butyl phosphine in dimethylformamide to release protein-bound homocysteine and to reduce mixed disulfides. The protein was precipitated with trichloroacetic acid and EDTA. After centrifugation at 9,500 × *g* for 5 minutes to remove the protein, the supernatant was treated with ammonium 7-flurobenzo-2-oxa-1,3-deazole-4-sulfonate to derivatize homocysteine and plasma thiols. The derivatized thiols were separated by high-performance liquid chromatography using a Beckman (Fullerton, CA) C18 pre-column and Beckman 5μ (4.55 mm × 25 cm) column. The metabolite levels were measured using a Hitachi (Tokyo, Japan) F-1050 fluorometer. An internal standard of cysteamine and standard curves were used to quantify the homocysteine. This methodology measures plasma tHcy, which includes the free homocystine and the protein-bound forms of homocysteine, as well as homocysteine-cysteine mixed disulfide. The intraassay coefficient of variation for this assay is 3%, whereas the interassay coefficient of variation is less than 7%. Plasma insulin was measured by a double-antibody radioimmunoassay (Ventrex Laboratories, Portland, MA).

Enzyme Activity Assays

CβS. The liver was immediately removed from the rats and frozen in liquid nitrogen until the assay was performed. CβS activity was measured by modification of the protocols of Mudd et al[14] and Kraus.[15] Briefly, liver tissue was homogenized in 0.05 mol/L KH_2PO_4 (pH 7.5) and centrifuged at 10,000 × *g* at 4°C and the supernatant was collected. The enzyme reaction mixture contained 0.1 mol/L Tris (pH 8.6), 1 mmol/L pyridoxal phosphate, 0.7 mg cellular protein extract, 0.5 mg/mL bovine serum albumin, 0.06 μCi [^{14}C]serine (New England Nuclear, Boston, MA), 10 mmol/L serine, 15 mmol/L L-homocysteine (prepared from L-homocysteine thiolactone; Sigma, St Louis, MO), and 1 mmol/L cystathionine. The reaction mixture was incubated for 3 hours at 37°C, during which time [^{14}C]serine was converted by CβS to [^{14}C]cystathionine. [^{14}C]cystathionine was separated from [^{14}C]serine by ascending paper chromatography using 2-propanol:formic acid: water 80:6:20 (vol/vol) as the mobile phase. [^{14}C]cystathionine was cut out and counted in scintillation fluid. We defined 1 U enzyme activity as that which formed 1 nmol cystathionine per hour at 37°C. Enzyme-specific activity was expressed as units per milligram of cellular protein. Protein content was determined using the Bio-Rad (Hercules, CA) protein reagent.

MTHFR. The MTHFR activity assay was modified from the procedure used by Mudd et al.[16] The liver tissue was homogenized in 0.25 mol/L sucrose and centrifuged at 50,000 × *g* for 1 hour, and the supernatant was collected. The enzyme reaction mixture contained the following: 0.18 mol/L KH_2PO_4 (pH 6.3), 3.6 mmol/L menadione bisulfite, 1.4 mmol/L EDTA, 7.2 mmol/L ascorbic acid, 178 μmol/L flavin adenine dinucleotide, and 420 μmol/L [^{14}C]-5-methyltetrahydrofolate. The reaction mix was incubated for 1 hour at 37°C and then terminated by the addition of 0.6 mol/L sodium acetate, pH 4.5, 0.1 mol/L formaldehyde, and 0.4 mol/L dimedone (5,5-dimethyl-1,3-cyclohexanedione) in 50% ETOH. The samples were boiled, toluene was added, and 2 mL of the upper phase containing [^{14}C]formaldehyde was removed for counting in scintillation fluid. The enzyme activity was expressed as nanomoles per hour formed per milligram of protein.

RNA Extraction and Northern and Slot Blot Analysis

Total cellular RNA was prepared using Tri Reagent (Molecular Research Center, Cincinnati, OH) by the method of Chomczynski and Sacchi.[17] The RNA was quantified by absorbance measurements at 260 nm and confirmed by analysis of rRNA bands on a gel.

The probes for each enzyme (CβS and MTHFR) were as follows: (1) a 1.55-kb cDNA encoding the human CβS cDNA[18] (provided by Dr Jan Kraus, University of Colorado Health Sciences Center) and (2) a 2.2-kb cDNA encoding the human MTHFR enzyme[19] (provided by Dr Rima Rozen, McGill University). The cDNA for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used to control for loading. Following appropriate restriction enzyme digestion of the insert-containing plasmids, the resulting cDNAs were gel-purified and labeled with [^{32}P]-dCTP (3,000 Ci/mmol; Dupont, Boston, MA) using a random primer labeling kit to a specific activity of about 3.6 × 10^6 cpm/ng DNA. Twenty micrograms of total cellular RNA was transferred to a nylon membrane (Bio-Rad) by slot blot using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad) in 10× SSC. The membrane was fixed, probed, and washed as described previously.[20]

The blot was then exposed to Kodak (Rochester, NY) BIOMAX MS film at −70°C with a BIOMAX MS intensifying screen for 24 hours. CβS and MTHFR message concentrations were quantified by densitometry using a Molecular Dynamics (Sunnyvale, CA) Laser Densitometer (model 300A). An arbitrary unit was designated as the ratio of message between CβS or MTHFR and GAPDH.

[^{14}C]-5-methyltetrahydrofolate and [^{14}C]cystathionine were purchased form Amersham (Piscataway, NJ), and [^{14}C]serine was from New England Nuclear (Boston, MA). All other chemicals were purchased from Sigma (St Louis, MO).

Statistics

Data were analyzed using Student's *t* test, with significance reported at a P level less than .05. Correlation and regression analysis was performed using the Sigmastat software program (Jandel Scientific, San Rafael, CA).

RESULTS

Rats that were fed the HFS and LFCC diets were killed at 6 months and 2 years of age. HFS rats were generally heavier than LFCC rats, and these differences were apparent at both 6 months and 2 years. In addition, the HFS rats were hyperinsulinemic, and plasma homocysteine concentrations were significantly higher in HFS rats compared with LFCC rats at both 6 months and 2 years (Table 1).

HFS rats had significantly lower levels of hepatic CβS activity compared with LFCC rats at both 6 months and 2 years. The 6-month-old and 2-year-old HFS animals had CβS activity levels that were 71% and 61% of their LFCC group, respectively (both $P < .05$). This decrease in CβS activity was reflected in a decrease of CβS mRNA concentrations. Three mRNA species were identified for MTHFR at about 4.4, 2.3, and 2.0 kb, and 2 species were observed for CβS, with a predominant band at about 2.4 kb and a lighter band at 4.0 kb. Previous studies have identified multiple mRNA species for CβS.[17] When CβS mRNA concentrations were quantified by slot-blotting, the CβS mRNA level in HFS animals was 70% of

Table 1. Body Weight and Plasma Insulin and tHcy of 6-Month-Old and 2-Year-Old Rats in HFS and LFCC Groups

Group	Weight (g)	Plasma Insulin (μU/mL)	Plasma tHcy (μmol/L)	MTHFR Activity (nmol/h mg protein)	MTHFR/ GAPD Ratio (arbitrary units)	CβS Activity (U/mg protein)	CβS/GAPD Ratio (arbitrary units)
6-Month-old							
HFS	214 ± 3.4*	56 ± 12*	10.8 ± 0.9*	8.6 ± 0.4*	1.7 ± 0.3*	0.45 ± .02*	1.0 ± 0.1
LFCC	188 ± 1.4	14.5 ± 2.9	6.9 ± 0.3	4.8 ± 0.3	1.0 ± 0.2	0.64 ± .03	1.3 ± 0.1
2-Year-old							
HFS	367 ± 9.4*	61.2 ± 16.5*	8.7 ± 0.4*	10.1 ± 0.2*‡	1.5 ± 0.1*	0.35 ± 0.03*‡	0.7 ± 0.03*‡
LFCC	254 ± 5.9	13.6 ± 1.4	5.3 ± 0.4†	5.5 ± 0.3	1.1 ± 0.2	0.57 ± 0.08	0.9 ± 0.1

NOTE. Values are reported as the mean ± SEM; n = 8 for each group.
*$P < .05$ *v* control.
†$P < .01$ *v* 6-month-old LFCC.
‡$P \leq .06$ *v* 6-month-old HFS.

the concentration in their respective LFCC rats at 2 years ($P < .05$).

The activity of MTHFR was measured in the liver homogenate. In contrast to the decrease in CβS, HFS rats demonstrated higher levels of MTHFR enzyme activity compared with LFCC rats. The increase in MTHFR activity in HFS animals compared with the LFCC controls was 179% and 183% at 6 months and 2 years, respectively (both $P < .05$).

Several different transcripts for MTHFR and CβS were observed. Because of the multiple mRNA species, we used a slot blot for quantitation. Comparisons are made between 6-month-old and 2-year-old HFS and LFCC rats for total liver RNA. At both 6 months and 2 years, HFS rats showed increased concentrations of MTHFR mRNA compared with the LFCC group. At 6 months, HFS rats demonstrated a 207% increase in MTHFR mRNA, and there was a 137% increase for 2-year-old HFS rats (both $P < .05$).

Age-Related Changes

In both HFS and LFCC rats, plasma homocysteine concentrations were lower in 2-year-old versus 6-month-old animals (6-month HFS *v* 2-year HFS, $P = .071$; 6-month LFCC *v* 2-year LFCC, $P = .008$). Although there were no age-related differences in CβS or MTHFR activity levels in LFCC rats, the older HFS rats demonstrated a tendency for lower CβS activity and higher MTHFR levels. However, the difference between the enzyme activity at 6 months and 2 years was not statistically significant.

As already described, HFS rats were hyperinsulinemic, and previous studies have demonstrated that rats fed high-sucrose diets are insulin-resistant. To determine whether the degree of hyperinsulinemia was related to the changes in CβS and MTHFR, we examined the relationship between the fasting insulin concentration and hepatic CβS and MTHFR activity levels. With linear regression, there were significant positive correlations between fasting insulin and plasma tHcy ($r = .51$, $P < .01$; Fig 1). HFS rats were hyperinsulinemic and also heavier than LFCC rats, and therefore there was a significant correlation between plasma tHcy and body weight ($r = .67$, $P < .01$). In addition to a significant correlation between fasting insulin and tHcy, there were also significant correlations between plasma insulin and MTHFR activity ($r = .48$, $P < .01$; Fig 2) and between plasma insulin and CβS activity ($r = -.54$, $P < .001$; Fig 3). Finally, there was a significant inverse relationship between CβS and MTHFR activities ($r = -.58$, $P < .001$; Fig 4).

DISCUSSION

Elevated plasma homocysteine concentrations usually occur as a result of inherited or acquired disorders that alter enzyme activity in the transsulfuration and remethylation pathways.[1] In the transsulfuration reaction, homocysteine irreversibly condenses with serine to form cystathionine. This irreversible reaction is catalyzed by the pyridoxine-dependent enzyme CβS. Cystathionine is subsequently hydrolyzed to cysteine. In the remethylation pathway, methionine reforms from homocysteine when a methyl group is donated by *N*-5-methyltetrahydrofolate. The enzyme MTHFR is responsible for the conversion of 5,10-methylenetetrahydrofolate to *N*-5-methylenetetrahydrofolate. Alternatively, the methyl group may be donated by betaine. The remethylation pathway is the primary determinant of the plasma homocysteine concentration in the fasting state, while

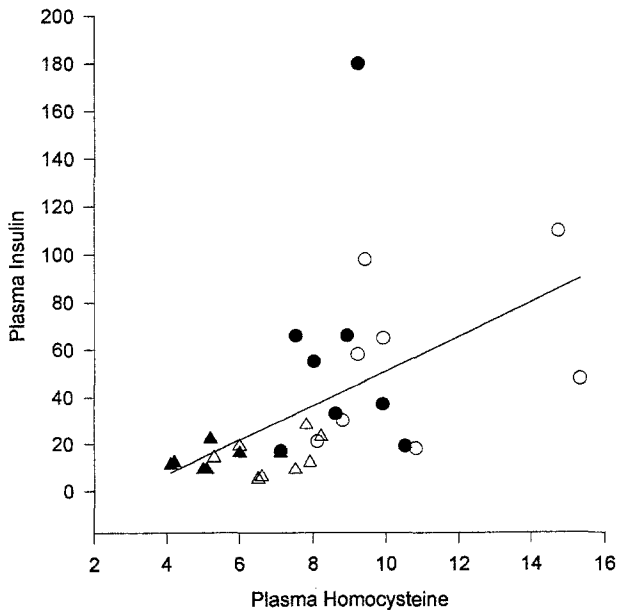

Fig 1. Relationship between plasma tHcy and plasma insulin (μU/mL) in rats fed HFS and LFCC diets. HFS rats at 6 months, ●; HFS rats at 2 years, ○; LFCC rats at 6 months, ▲; LFCC rats at 2 years, △.

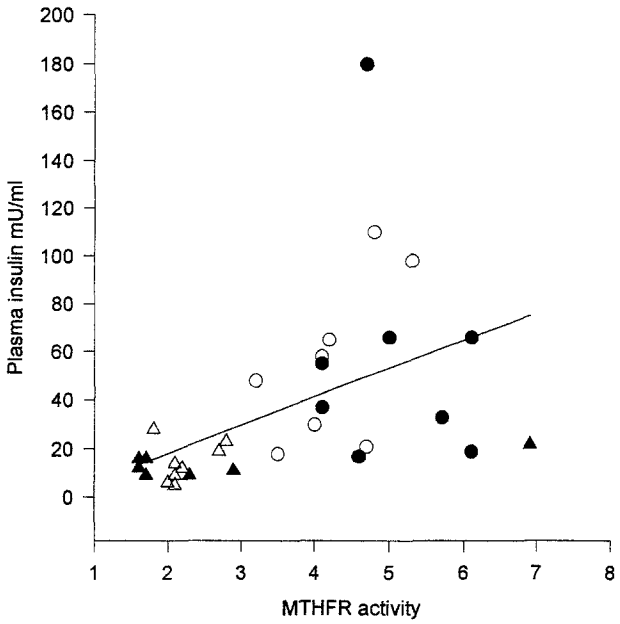

Fig 2. Relationship between plasma insulin and MTHFR activity (U/mg protein) in rats fed HFS and LFCC diets. HFS rats at 6 months, ●; HFS rats at 2 years, ○; LFCC rats at 6 months, ▲; LFCC rats at 2 years, △.

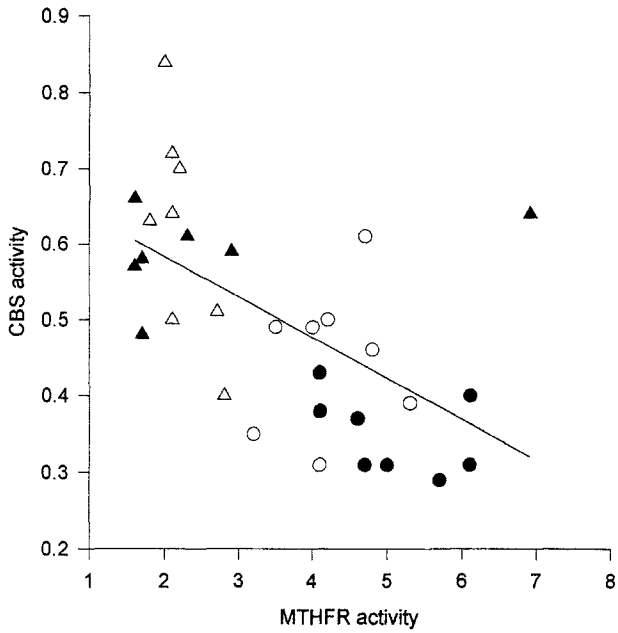

Fig 4. Reciprocal relationship between CβS and MTHFR activity (both U/mg protein) in the liver in rats fed HFS and LFCC diets. HFS rats at 6 months, ●; HFS rats at 2 years, ○; LFCC rats at 6 months, ▲; LFCC rats at 2 years, △.

the transsulfuration pathway is most active following a methionine load.

Partial defects of the enzymes in homocysteine metabolism are well recognized. Individuals with a partial defect in CβS activity, such as heterozygote parents of homocystinuric children, have normal fasting plasma homocysteine concentrations but abnormal methionine-load tests.[1,21] Alternatively, nutritional deficiencies of cobalamin (vitamin B_{12}), folate, or pyridoxine (vitamin B_6) can result in blockade/dysfunction of the homocysteine metabolic pathways, because the activity of the enzymes depends on these vitamins as cofactors. Recent evidence suggests that even in the presence of a genetic mutation of MTHFR, environmental factors such as nutritional status (folic acid intake) can exert some influence on plasma homocysteine concentrations.[22-25]

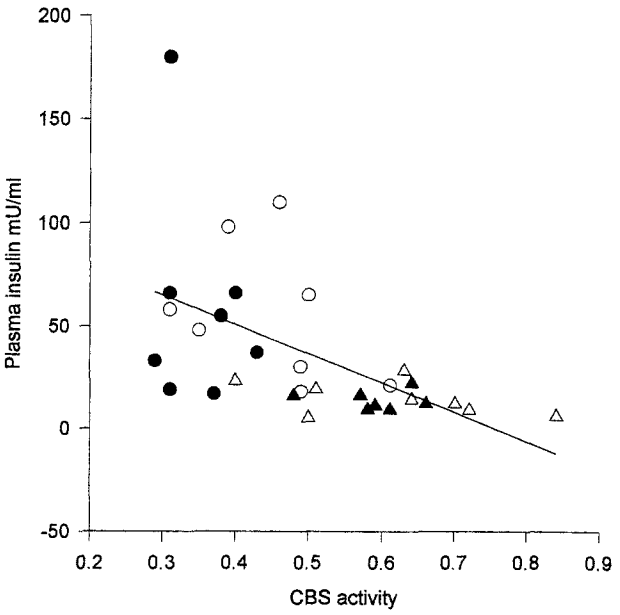

Fig 3. Relationship between plasma insulin and CβS activity (U/mg protein) in rats fed HFS and LFCC diets. HFS rats at 6 months, ●; HFS rats at 2 years, ○; LFCC rats at 6 months, ▲; LFCC rats at 2 years, △.

In this study, we wished to determine whether insulin resistance in rodents was associated with HH and, more importantly, to examine the expression of the key enzymes in homocysteine metabolism. Although several enzymes are involved in methionine-homocysteine metabolism, we chose to study MTHFR and CβS in this model because abnormalities of these 2 enzymes have been most frequently associated with hyperhomocysteinemia in humans.

Our data demonstrate that hyperinsulinemic HFS rats had decreased CβS activity and increased MTHFR activity. Because HFS rats are hyperinsulinemic but have normal blood glucose concentrations,[6] these data suggest that the changes in CβS and MTHFR are not due to diabetes, but may be due to the insulin resistance or hyperinsulinemia. Because these changes in activity were accompanied by similar changes in enzyme mRNA concentrations, these data suggest that these enzymes are regulated transcriptionally, or at the level of mRNA processing. Thus, some component of carbohydrate metabolism may have a significant role in the regulation of gene expression of these key enzymes in homocysteine metabolism. Whether this regulation is due to the elevated blood insulin, insulin resistance, increased glucose transport, or some other factor has yet to be determined. In previous studies, the HFS rat has been shown to have hypertension and elevated plasma triglycerides. Further studies are needed to determine whether these factors contribute to HH.

It is not clear why the metabolic changes induced by the HFS diet should have differential effects on CβS and MTHFR. Since both CβS and MTHFR are involved in homocysteine clearance and metabolism, one explanation could be that direct influences on one enzyme resulted in a compensatory effect of the other in an attempt to keep plasma homocysteine at a relatively constant level. For example, hyperinsulinemia may have caused a decrease in CβS, and the resulting increase in tHcy caused an increase in MTHFR, which would tend to prevent extreme elevations in plasma tHcy. In support of such a compensatory response, we have observed a significant negative correlation between CβS and MTHFR activities in these animals. However, an increase in plasma homocysteine was observed, suggesting that such compensatory changes were either incomplete or insufficient. Another possible explanation for the elevated plasma homocysteine involves enzyme levels in other tissues. We have measured CβS and MTHFR only in the liver. However, these enzymes are also expressed in other tissues such as the kidney, where MTHFR may have an important role.[26]

Jacobs et al[27] investigated homocysteine metabolism in a type 1 diabetic animal model (streptozotocin-treated rats) to examine whether insulin plays a role in its regulation. Plasma homocysteine was lower in the untreated diabetic rat, but this decrease in homocysteine was prevented when diabetic rats received insulin. They also found an increase in the activity of the hepatic (but not renal) transsulfuration enzymes (CβS and cystathionine-γ-lyase) in the untreated diabetic rat, which was normalized by insulin treatment. These results suggest that insulin is involved in the regulation of plasma homocysteine concentrations by affecting the hepatic transsulfuration pathway, which is involved in the catabolism of homocysteine. The differences between our data and those of Jacobs et al may relate to changes in body weight, as well as differences in the animal models for type 1 and type 2 diabetes.

These studies have a number of implications for humans. Both insulin resistance and HH are independent risk factors for cardiovascular disease, and recent studies suggest that these risk factors may coexist in some patients.[5,28] Although diabetic patients have relatively normal fasting homocysteine concentrations, patients with type 2 diabetes and macrovascular disease have significantly elevated plasma homocysteine following a methionine load.[5] Although there are no mechanistic data in humans, such an abnormality would be suggestive of a defect in the transsulfuration pathway, where CβS is the key enzyme, rather than the remethylation pathway, which is dependent on MTHFR.[2] Although we did not study these rats after a methionine load, the primary enzyme defect in the experiments was in CβS. Nevertheless, insulin resistance is a complex condition in humans, and it is impossible to determine whether insulin resistance causes HH or whether HH is due to some other defect associated with insulin resistance.

Although it is not known whether insulin-resistant or diabetic humans have abnormalities in CβS or MTHFR, even subtle defects in these enzymes may be important in subjects with marginal nutritional status. For example, subjects who are heterozygous for the common mutation in MTHFR (thermolabile form) have elevated plasma tHcy only when they have folate deficiency.[23-25] Thus, the combination of nutritional inadequacies along with changes in carbohydrate metabolism could result in elevated plasma tHcy, which could further increase the risk for atherosclerosis in diabetic subjects. However, our study was performed in rodents, and further investigation is necessary before these conclusions can be extrapolated to humans.

We conclude that HFS rats, which demonstrate obesity and hyperinsulinemia, demonstrate elevated plasma tHcy along with reciprocal changes in 2 key enzymes in tHcy metabolism: CβS and MTHFR. In addition, these 2 enzymes appear to be regulated under these conditions in a reciprocal manner, and the degree of change is correlated with the degree of obesity and hyperinsulinemia.

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