Effects of Streptozotocin-Induced Diabetes and of Insulin Treatment on Homocysteine Metabolism in the Rat

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An elevation in the concentration of total plasma homocysteine is known to be an independent risk factor for the development of vascular disease. Alterations in homocysteine metabolism have also been observed clinically in diabetic patients. Patients with either type 1 or type 2 diabetes who have signs of renal dysfunction tend to exhibit elevated total plasma homocysteine levels, whereas type 1 diabetic patients who have no clinical signs of renal dysfunction have lower than normal plasma homocysteine levels. The purpose of this study was to investigate homocysteine metabolism in a type 1 diabetic animal model and to examine whether insulin plays a role in its regulation. Diabetes was induced by intravenous administration of 100 mg/kg streptozotocin to Sprague-Dawley rats. We observed a 30% reduction in plasma homocysteine in the untreated diabetic rat. This decrease in homocysteine was prevented when diabetic rats received insulin. Transsulfuration and remethylation enzymes were measured in both the liver and the kidney. We observed an increase in the activities of the hepatic transsulfuration enzymes (cystathionine β-synthase and cystathionine γ-lyase) in the untreated diabetic rat. Insulin treatment normalized the activities of these enzymes. The renal activities of these enzymes were unchanged. 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. *Diabetes* 47:1967–1970, 1998

Homocysteine, a thiol-containing nonprotein amino acid, is an intermediate in the metabolism of methionine. Elevated total plasma homocysteine has been recognized as an independent risk factor for the development of vascular diseases (1,2). Several factors are known to affect plasma homocysteine levels, among them certain vitamin deficiencies. Folate and vitamin B-12 are required for methionine synthase activity, which catalyzes the remethylation of homocysteine to methionine, whereas vitamin B-6 is required for both enzymes of the transsulfuration pathway (cystathionine β-synthase and cystathionine γ-lyase). Both folate and vitamin B-12 levels have been shown to correlate negatively with plasma homocysteine levels (3,4). However, adverse effects of vitamin B-6 deficiency on homocysteine levels have not yielded consistent results (5,6). Increased homocysteine concentrations are also present in genetic disorders such as cystathionine β-synthase deficiency, in which homocysteine cannot be converted to cystathionine.

Cardiovascular diseases are the major cause of death in diabetic patients (7), and there has recently been much interest in the possible role of homocysteine in the development of cardiovascular diseases in these patients. Homocysteine levels in human diabetic patients appear to depend on the presence or absence of nephropathy. Diabetic patients with elevated creatinine levels (an indicator of kidney dysfunction) tend to exhibit an increase in plasma homocysteine (8–10). This is consistent with studies that show that the kidney is an important organ in the removal of plasma homocysteine (11). On the other hand, type 1 patients with normal creatinine levels have decreased plasma homocysteine (12). The reason for such decreased homocysteine levels is unknown, but the regulation of homocysteine metabolism by insulin could afford an explanation. The purpose of this study was to examine homocysteine metabolism in streptozotocin-induced diabetic rats and to determine whether insulin may regulate this metabolism.

RESEARCH DESIGN AND METHODS

Animals, treatments, and tissue sampling. All procedures were approved by Memorial University's Institutional Animal Care Committee and are in accordance with the guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (supplied from our university's breeding colony) weighing 280–350 g were used in all studies. Animals were placed in individual metabolic cages and exposed to a 12-h light/dark cycle beginning with light at 0800. The animals ate an AIN-93 diet ad libitum and had free access to water. Diabetes was induced by a single intravenous injection of 100 mg/kg streptozotocin (dissolved in 10 mmol/l citrate buffer [pH 4.5] immediately before use) under light ether anesthesia, and an equal volume of citrate buffer was administered to control rats. Half of the diabetic rats, the "untreated diabetic" group, were treated with Novolin ultralente human insulin (Eli Lilly, Indianapolis, IN) subcutaneously for 5 days, after which insulin was withdrawn and the rats received saline injections for 5 days ($n = 8$). The remaining diabetic rats, the "insulin-treated diabetic" group, received insulin injections for the entire 10 days of the experiment ($n = 8$). The control group received saline injections ($n = 8$). Insulin was administered at 0900 and 1900; the dose was adjusted to maintain blood glucose close to control values as measured with an Ames Glucometer II (Miles Canada, Etobicoke, Ontario, Canada), using a drop of blood obtained by tail prick.

At 1030 on the day of the study, animals were anesthetized with 65 mg/kg i.p. sodium pentobarbital. After a midline abdominal incision was made, urine and blood samples were collected from the bladder and abdominal aorta, respectively. Kidneys and liver were then rapidly removed and placed in ice-cold 50 mmol/l potassium phosphate buffer (pH 6.9). Heparinized tubes containing the blood samples were placed on ice until they were centrifuged for 15 min, using a clinical centrifuge (3,700*g*) for plasma separation. The plasma and urine were

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HPLC, high-performance liquid chromatography.

TABLE 1
Body weight, food intake, and plasma glucose and creatinine concentrations in control, untreated diabetic, and insulin-treated diabetic rats

	Control	Untreated diabetic	Insulin-treated diabetic
Initial weight (g)	316 ± 17[a]	318 ± 18[a]	313 ± 23[a]
Final weight (g)	365 ± 22[c]	275 ± 15[a]	313 ± 27[b]
Food intake (g/day)	27 ± 4.1[a]	30 ± 9.4[a]	28 ± 4.0[a]
Plasma glucose (mmol/l)	7.6 ± 1.2[a]	34.8 ± 3.5[b]	7.4 ± 1.7[a]
Plasma creatinine (μmol/l)	81 ± 5.9[b]	61 ± 4.8[a]	79 ± 7.2[b]

Data are means ± SD. Food intake was determined daily. Glucose and creatinine measurements were made on day 10, i.e., at the end of the experimental period. Differences in superscript letters within rows signify differences ($P < 0.05$) between groups.

frozen (–20°C) for later use. Fresh tissues were diluted 1:5 with 50 mmol/l phosphate buffer, then homogenized with a Polytron (Brinkmann Instruments, Toronto, Canada) for 20 s at 50% output. The homogenates were centrifuged at 18,000*g* at 4°C for 30 min, and the supernatant was retained. All enzyme assays were carried out on this 18,000*g* (postmitochondrial) supernatant.

Analytical procedures. Methionine synthase activity was measured on fresh samples by a radioactive assay described by Kolbin et al. (13). Betaine:homocysteine methyltransferase activity was determined using an assay by Wang et al. (14). Cystathionine β-synthase was assayed on previously frozen samples using the method of Miller et al. (15). For the betaine:homocysteine methyltransferase activity and cystathionine β-synthase assays, methionine and cystathionine, respectively, were measured by high-performance liquid chromatography (HPLC) (16). Cystathionine γ-lyase activity was determined on previously frozen samples following the method of Stipanuk (17). Methylenetetrahydrofolate reductase was measured by a radioactive assay described by Engbersen et al. (18). Protein concentration was determined using the biuret method (19), after solubilization with deoxycholate, using bovine serum albumin as a standard (20). All enzyme assays were demonstrated to be linear with respect to time and protein concentrations. Enzyme activities are expressed as nanomoles of product produced per milligram protein per minute. Homocysteine concentrations were determined using HPLC by the method of Vester and Rassmussen (21). Plasma glucose (22) and creatinine (Sigma kit) concentrations were determined by standard methods. Homocysteine excretion was estimated from the homocysteine concentration of the bladder urine and 24-h urinary volume as measured in metabolic cages.

Statistical analysis. Data are presented as means ± SD. Analysis of variance was used to compare data, and the Mann-Whitney *U* test was performed to assess differences between group means. A value of $P < 0.05$ was chosen to represent a significant difference.

Chemicals. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted.

RESULTS

Table 1 gives information on body weight, food intake, plasma glucose, and plasma creatinine. Although the rats' final weights differed, there were no differences in the food intake between groups. Given that the groups consumed similar quantities of food, we cannot attribute alterations in homocysteine metabolism to differences in vitamin or amino acid intake. The plasma glucose in the untreated diabetic rat indicated frank diabetes, which was well controlled by insulin in the treated diabetic group. Plasma creatinine levels in the untreated diabetic rats were not elevated; in fact, they were slightly lower than the other groups, providing clear evidence that renal function was not impaired in the diabetic rats. In a separate study, net renal homocysteine removal was unaltered in the diabetic rats (data not shown), so that possible effects of glomerular hyperfiltration cannot account for the differences in plasma homocysteine concentrations.

Plasma homocysteine levels in the streptozotocin-induced diabetic rats were ~40% lower than those in the controls (untreated diabetic versus control rats: 6.97 ± 1.17 vs. 10.91 ± 1.14 μmol/l [$P < 0.05$]) (Fig. 1). Insulin treatment prevented the decrease in the plasma homocysteine concentrations in diabetic rats (10.33 ± 0.56 μmol/l). This finding suggests that insulin may be involved in regulating plasma homocysteine levels.

We measured urinary homocysteine to determine whether the decreased plasma homocysteine in diabetes could be due to increased urinary loss. There was no difference among the three groups in the quantity of homocysteine excreted per day (0.59 ± 0.19, 0.53 ± 0.08, and 0.43 ± 0.08 μmol/day in the control, untreated diabetic, and insulin-treated groups, respectively). Bostom et al. (11) showed that only 2% of the total homocysteine removed by the kidney appears in the urine. Thus, homocysteine excretion does not represent a major route of homocysteine loss from the body, and it is not increased by untreated diabetes.

The liver is a major organ in methionine metabolism, as evidenced by the distribution and specific activities of the enzymes of methionine metabolism as well as by its role in creatine synthesis, the main methyl-requiring reaction in the

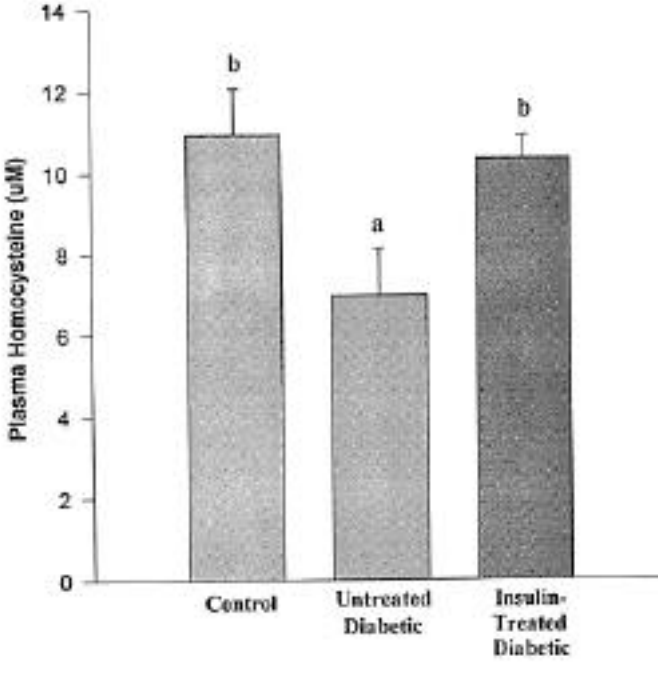

FIG. 1. Comparison of total plasma homocysteine in control, untreated diabetic, and insulin-treated diabetic rats. Data are expressed as means ± SD. Differences in letters (*a, b*) between columns signify differences ($P < 0.05$) between groups.

TABLE 2
Hepatic activities of enzymes of homocysteine metabolism in control, untreated diabetic, and insulin-treated diabetic rats

	Control	Untreated diabetic	Insulin-treated diabetic
Cystathionine β-synthase	0.70 ± .10[a]	0.98 ± 0.31[b]	0.68 ± 0.14[a]
Cystathionine γ-lyase	10.4 ± 1.5[a]	22.5 ± 0.9[b]	10.0 ± 1.3[a]
Methionine synthase	0.077 ± 0.011[a]	0.073 ± 0.005[a]	0.084 ± 0.018[a]
Betaine:homocysteine methyltransferase	3.2 ± 0.3[a]	4.1 ± 1.4[a]	2.9 ± 1.0[a]
Methylenetetrahydrofolate reductase	0.33 ± 0.06[a]	0.26 ± 0.07[a]	0.25 ± 0.05[a]

Data are means ± SD. Enzyme activity is expressed in nanomoles per milligram of protein per minute. Differences in superscript letters within rows signify differences ($P < 0.05$) between groups.

body (23). In light of this fact, it is likely, although it has not been proved, that the liver is the major producer of plasma homocysteine. Svardal et al. (24) have shown that hepatocytes release homocysteine into their culture medium. The kidney appears to be the major organ of homocysteine removal (10). We, therefore, measured the activities of the enzymes in the transsulfuration and remethylation pathways in both the liver and the kidney.

In the untreated diabetic rats, there was a 30% increase in cystathionine β-synthase activity, and cystathionine γ-lyase activity in liver was elevated twofold (Table 2). Insulin treatment prevented such an increase in the activities of these enzymes. No difference was observed in the activities of methionine synthase, methylenetetrahydrofolate reductase, and the betaine:homocysteine methyltransferase, suggesting that the reduction of plasma homocysteine was not the result of an activation of one of the remethylation pathways. These data suggest the importance of the hepatic transsulfuration pathway and of the role of insulin in homocysteine metabolism. Our earlier work showed that the hepatic level of methionine is markedly reduced in diabetic rat liver, whereas the hepatic level of cysteine is increased threefold, which is consistent with increased flux through the transsulfuration pathway in the diabetic liver (25). In addition, Watanabe et al. (26) have shown that transgenic mice that are heterozygous for cystathionine β-synthase deficiency (i.e., that express only one functional cystathionine β-synthase allele) have only half the normal activity of this enzyme and twice the normal plasma homocysteine levels. These findings serve as good evidence for the key role of this enzyme in the regulation of plasma homocysteine concentrations.

Unlike the liver, the kidney showed no change in the activity of the transsulfuration enzymes (Table 3). A decrease in renal methionine synthase activity was observed and was corrected by insulin treatment. The renal activity of methylenetetrahydrofolate reductase was decreased in the diabetic rats, but it was not corrected by insulin treatment. Betaine:homocysteine methyltransferase is not found in the rat kidney (27).

DISCUSSION

The mechanism by which insulin, or possibly one of its counterregulatory hormones, regulates the enzyme activities of the hepatic transsulfuration pathway is unknown. It is possible that the genes for both enzymes are regulated by insulin and/or counterregulatory hormones. If insulin acted as the regulatory agent, it might do so by repressing these enzymes, as it does, for example, for phosphoenolpyruvate carboxykinase (28). On the other hand, the enzymes may be induced by counterregulatory hormones. It is known that cystathionine β-synthase may be induced in rat hepatoma cells by a combination of dexamethasone and agents that elevate cAMP (29). Given that glucagon acts by increasing cellular cAMP levels, it is possible that it plays a role, together with glucocorticoids, in the induction of this enzyme. Of course, it is also possible that the increased activities of the two enzymes of the transsulfuration pathway are due to the activation of these enzymes rather than to their induction. Little is known about possible metabolic regulation of cystathionine γ-lyase. However, the liver is known to contain two forms of cystathionine β-synthase: a tetramer of 63-kDa subunits and a dimer of 48-kDa subunits. The 48-kDa polypeptide arises through the proteolytic cleavage of the 63-kDa polypeptide (30). The smaller form of the enzyme has been shown to have a much lower K_m for homocysteine, thus presenting a potential site for regulation (30). Further work is necessary to determine which, if any, of these mechanisms bring about the observed changes in enzyme activity.

It is clear that there is a decrease in plasma homocysteine in type 1 diabetes. This study is the first to shed some light on

TABLE 3
Renal activities of enzymes of homocysteine metabolism in control, untreated diabetic, and insulin-treated diabetic rats

	Control	Untreated diabetic	Insulin-treated diabetic
Cystathionine β-synthase	0.36 ± 0.05[a]	0.35 ± 0.08[a]	0.35 ± 0.09[a]
Cystathionine γ-lyase	6.2 ± 1.1[a]	6.4 ± 1.9[a]	5.8 ± 1.4[a]
Methionine synthase	0.34 ± 0.03[b]	0.25 ± 0.01[a]	0.36 ± 0.04[b]
Methylenetetrahydrofolate reductase	0.79 ± 0.07[b]	0.24 ± 0.08[a]	0.33 ± 0.06[a]

Data are means ± SD. Enzyme activity is expressed in nanomoles per milligram of protein per minute. Differences in superscript letters within rows signify differences ($P < 0.05$) between groups.

the possible metabolic mechanisms responsible for such a change. The decrease in plasma homocysteine levels was associated with an increase in the enzymatic activities of the hepatic transsulfuration pathway. Normalization of these parameters after insulin treatment emphasizes the importance of insulin in homocysteine metabolism. This study offers the first demonstration that insulin has a possible role in the metabolic regulation—and, through its effects on the transsulfuration pathway, in the metabolic disposal—of this highly atherogenic amino acid. The mode of insulin's action requires further study.

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