Plasma Homocysteine Concentrations Are Regulated by Acute Hyperinsulinemia in Nondiabetic But Not Type 2 Diabetic Subjects

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An association between hyperhomocysteinemia and premature atherosclerosis in patients with non–insulin-dependent diabetes mellitus (NIDDM) has recently been described. Little is known about the role of insulin in homocysteine [H(e)] metabolism. We measured plasma H(e) concentrations in the fasting state and during a hyperinsulinemic-euglycemic clamp in normal subjects and patients with NIDDM. Plasma H(e) decreased significantly from 7.2 ± 2.6 to 6.0 ± 2.7 mmol/L ($P < .01$) in normal subjects, but did not change in patients with NIDDM (6.0 ± 2.7 to 5.9 ± 2.5 mmol/L, respectively). These data suggest that plasma H(e) concentrations are regulated by acute hyperinsulinemia in normal subjects, but not in insulin-resistant NIDDM subjects. These abnormalities may have implications for the pathogenesis of premature vascular disease associated with NIDDM.

HOMOCYSTINURIA is caused by mutations in one of several enzymes involved in the metabolism of methionine and homocysteine [H(e)] and is associated with markedly accelerated atherosclerosis. Although homocystinuria is rare, a moderate elevation of plasma H(e) is an important risk factor for the development of premature cardiovascular disease.[1]

We recently described the association between an elevated plasma H(e) concentration following a methionine load and accelerated vascular disease in patients with non–insulin-dependent diabetes mellitus (NIDDM).[2] Elevated plasma H(e) concentrations have also been described in another study of patients with NIDDM, and it was possible to reduce plasma H(e) with vitamin B_{12} treatment.[3]

The most common enzyme defect in homocysteinemia involves cystathionine β-synthase (CBS). The frequency of mutations of CBS in the general population is less than 1%. Since plasma H(e) levels were moderately elevated in greater than 30% of our diabetic subjects with vascular disease,[2] it is unlikely that a major genetic mutation in CBS is responsible for the hyperhomocysteinemia. In recent studies, mutations of another enzyme, methylene tetrahydrofolate reductase (MTHFR), which is responsible for remethylation of H(e) to methionine, have been described. In particular, a thermolabile form of MTHFR is thought to be responsible for a large proportion of cases of hyperhomocysteinemia associated with vascular disease.[1] Since hyperhomocysteinemia seems to occur with a greater frequency in patients with NIDDM and vascular disease,[2,4] it is possible that metabolic abnormalities associated with NIDDM may contribute to hyperhomocysteinemia.

Insulin resistance is also a risk factor for cardiovascular disease.[5] Patients with the insulin resistance syndrome are resistant not only to insulin's action on glucose metabolism but also to its action on a number of other important cardiovascular factors, including vasodilatation[6] and platelet aggregation.[7]

It is well recognized that insulin has powerful effects on many aspects of protein and amino acid metabolism,[8] but its effect on H(e) metabolism has not previously been evaluated. We therefore studied the effect of acute hyperinsulinemia on plasma H(e) concentrations during a hyperinsulinemic-euglycemic glucose clamp.

SUBJECTS AND METHODS

Subjects

Fourteen normal subjects and 15 patients with NIDDM undergoing a hyperinsulinemic-euglycemic clamp for assessment of insulin sensitivity were included in the study. Clinical details of the patients and control subjects including lipid profiles and blood pressure are summarized in Table 1. All subjects had stable body weight for 6 months prior to the study. All consumed a standard weight-maintenance diet of 55% carbohydrate, 30% fat, and 15% protein over the 24 hours before the clamp study. The mean age was lower for the control subjects than for the patients.

None of the control subjects or patients were taking vitamin supplements, although one patient was taking ginseng extract. Seven diabetic patients were on treatment with diet alone, six were taking sulfonylureas, and two were taking insulin. Diabetic control in the patients was poor, with a fasting plasma glucose of 181 ± 16 mg/dL and hemoglobin A_{1C} (HbA_{1c}) of 9.9% ± 0.68%.

None of the patients or control subjects had clinical evidence of coronary artery disease in that there was no history of myocardial infarction or angina and electrocardiograms were normal in all of them. One normal subject and one diabetic patient were on treatment for hypertension with an angiotensin-converting enzyme inhibitor. One diabetic patient was on a combination of pravastatin and gemfibrozil for hyperlipidemia. The only female diabetic patient in the study was 50 years old and taking Premarin (Wyeth-Ayerst) as hormone replacement therapy. Both female control subjects were premenopausal.

Blood samples were obtained before the clamp and 120 to 180 minutes after induction of hyperinsulinemia. Two samples were obtained during the clamp, and the mean H(e) level of the two samples was used in subsequent analysis of the data. The coefficient of variation of this assay is less than 5%.

Plasma H(e) levels were measured using a modification of the method described by Ubbink et al.[9] Blood was collected and immediately centrifuged at 2,000 × *g* for 15 minutes at 20°C. The supernatant plasma was stored frozen at −70°C. The plasma was thawed and treated with tri-*n*-butyl phosphine in dimethylformamide (DMFA) to release protein-bound H(e) and to accomplish the reduction of mixed disulfides. Protein was precipitated with trichloroacetic acid and EDTA. After

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Table 1. Clinical Characteristics of the Subjects

Characteristic	Nondiabetic Controls	NIDDM Patients
Age (yr)	41.9 ± 5.8	52.1 ± 4.0
Gender (male:female)	8:2	14:1
Body mass index (kg/m^2)	30.7 ± 5.3	32.7 ± 3.7
Nonsmokers (n)	5	5
Waist to hip ratio	0.94 ± 0.07	0.97 ± 0.05
Fasting plasma glucose (mg/dL)	90.1 ± 8.8	180.5 ± 63.8*
HbA_{1c} (%)	5.3 ± 0.19	9.9 ± 0.68*
Fasting plasma insulin (μU/mL)	7.1 ± 9.1	17.3 ± 8.9*
Cholesterol (mg/dL)	189 ± 8	219 ± 12
Triglycerides (mg/dL)	131 ± 22	240 ± 31*
HDL (mg/dL)	41 ± 4	33 ± 2*
Systolic blood pressure	113 ± 2	126 ± 3*
Diastolic blood pressure	66 ± 3	77 ± 2*
Glucose disposal rate (mg/kg/min)	8.4 ± 3.6	4.5 ± 2.5*
Basal H(e) (μmol/L)	7.2 ± 2.6	6.0 ± 2.7
Clamp H(e) (μmol/L)	6.0 ± 2.7†	5.9 ± 2.5

*$P < .01$ *v* controls.
†$P < .01$ *v* basal H(e).

centrifugation at 9,500 × *g* for 5 minutes to remove protein, the supernatant was treated with ammonium 7-flurobenzo-2-oxa-1,3 deazole-4-sulfonate to derivatize H(e) and plasma thiols. Derivatized thiols were separated on a Beckman C18 precolumn and Beckman 5u (4.55 mm × 25 cm) column (Beckman Instruments, Fullerton, CA) and measured using a Hitachi F-1050 fluorimeter (Hitachi Instruments, Danbury, CT). An internal standard of cysteamine and standard curves were used to measure H(e) levels. This methodology measures total plasma H(e), which includes the free and protein-bound forms of H(e).

Glucose Clamp Study

After basal measurements were obtained, a primed constant infusion of insulin (crystalline human insulin [Novolin R]; Novo Nordisk Pharmaceuticals, Princeton, NJ) was infused at submaximal and maximal rates of 40 and 300 mU/m^2/min, respectively, for measurement of glucose disposal. Seven nondiabetic and eight NIDDM patients were studied at an insulin infusion rate of 40 mU/m^2/min, and seven in each group were evaluated at 300 mU/m^2/min. KCl was infused at a rate of 0.16 mmol/min to avoid hypokalemia, and somatostatin was infused at a rate of 0.08 pmol/kg/min to suppress endogenous insulin secretion. During the entire clamp study, plasma glucose was maintained at euglycemia (90 to 95 mg/dL) by a variable infusion of exogenous 20% dextrose based on 5-minute arterialized plasma glucose concentrations.[10] Plasma glucose specific activity was measured every 10 to 20 minutes throughout the entire study for calculation of glucose disposal as previously described.[11,12]

Statistics

A paired *t* test was used to compare plasma H(e) levels at the beginning and end of the clamp study. Results are presented as the mean ±SD.

RESULTS

Clinical characteristics of the patients and nondiabetic control subjects are summarized in Table 1. Figures 1 and 2 illustrate the changes in plasma H(e) concentrations in individual patients and subjects before and during the clamp. The mean plasma H(e) concentration in normal subjects was 7.2 ± 2.6 mmol/L. During the clamp study, plasma H(e) concentrations decreased significantly to 6.2 ± 2.2 mmol/L ($P < .01$). In contrast, the mean plasma H(e) concentration in patients with NIDDM was 6.0 ± 2.7 mmol/L at baseline and was unchanged by hyperinsulinemia, with a level of 5.9 ± 2.5 mmol/L during the clamp (P = NS). There was no difference in the change of plasma H(e) when either 40 or 300 mU/m^2/min insulin was infused in either group.

The response of plasma H(e) to insulin was heterogeneous in nondiabetic subjects, with a decrease greater than 20% in five subjects and little change in three. In contrast, none of the patients with NIDDM had a decrease greater than 20% in plasma H(e) during the hyperinsulinemic clamp.

The glucose disposal rate in control subjects was 8.4 ± 3.6 mg/kg/min, significantly higher than in patients with NIDDM (4.5 ± 2.5 mg/kg/min). However, there was no correlation between the change in plasma H(e) and the glucose disposal

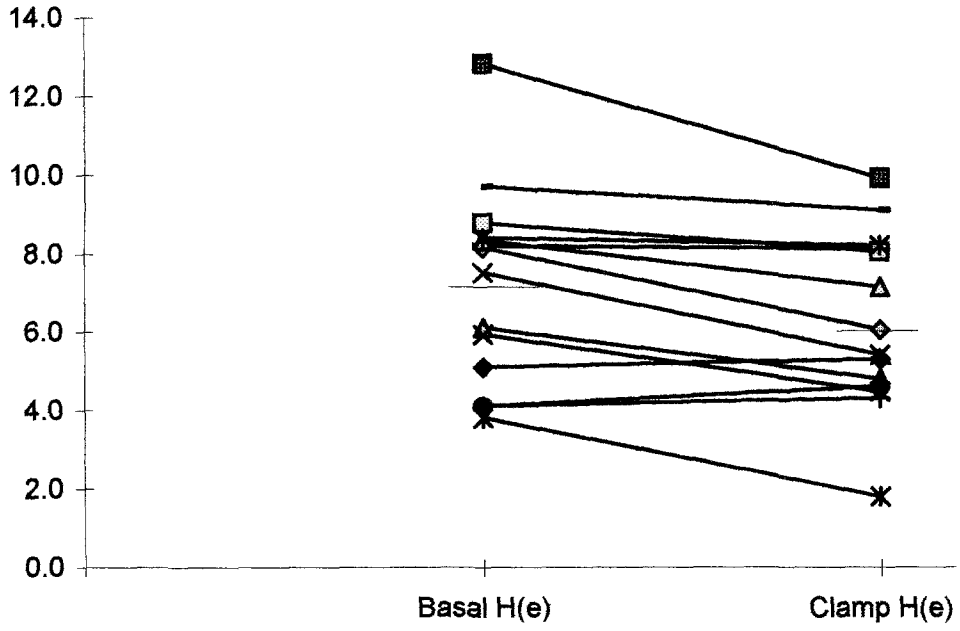

Fig 1. Plasma H(e) before and during a hyperinsulinemic clamp in normal subjects.

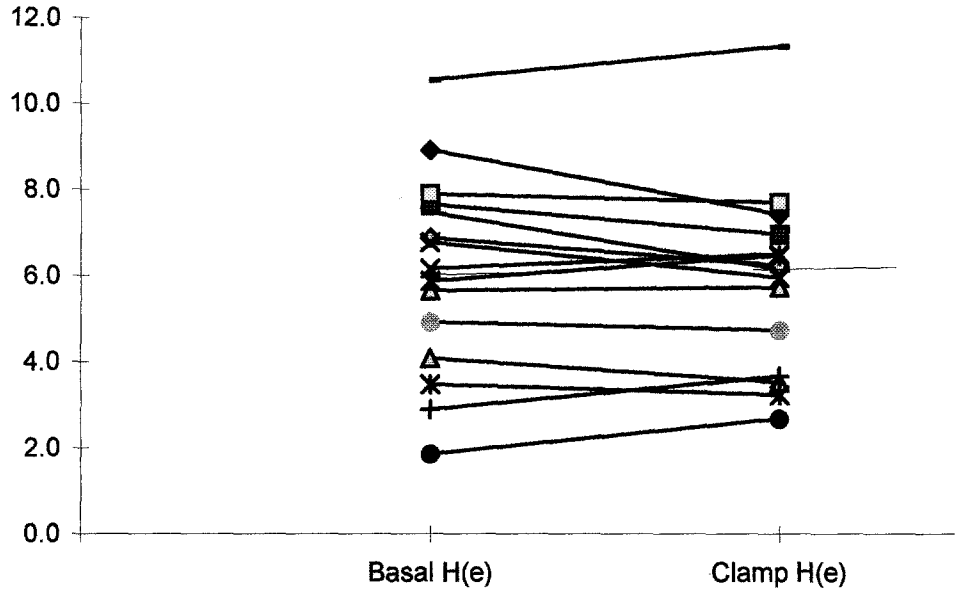

Fig 2. Plasma H(e) before and during a hyperinsulinemic clamp in NIDDM patients.

rate. Similarly, although plasma triglycerides and high-density lipoprotein (HDL) concentrations and systolic and diastolic blood pressure were significantly higher in patients compared with controls, there was no correlation between these parameters and changes in plasma H(e) during the clamp.

DISCUSSION

Our data demonstrate that insulin plays a role in normal H(e) metabolism. Acute hyperinsulinemia results in a significant decrease of plasma H(e) in nondiabetic subjects. However, in insulin-resistant subjects with NIDDM, there was no change in H(e) during either the high- or low-dose clamp. These data suggest that resistance to the effects of insulin on glucose disposal may be associated with resistance to the suppressive effect of insulin on H(e) levels in patients with NIDDM. A resistance to insulin's effect on H(e) may contribute to the increased cardiovascular disease associated with the insulin resistance syndrome and NIDDM.

We did not observe a dose-dependent effect of insulin on plasma H(e), possibly because the maximum effect of insulin occurs at a physiological level. It has been suggested that some elements of protein metabolism, such as proteolysis, are less sensitive to insulin than lipolysis or glucose production.[8] There was no correlation between the change in plasma H(e) and the glucose disposal rate. The glucose disposal rate during a clamp is an indicator of insulin sensitivity in terms of insulin's effect on glucose metabolism. The number of patients in our study is small and may be insufficient to determine whether there was a true relationship between insulin's effect on glucose and protein metabolism.

Our patient population was heterogenous in terms of the treatment of diabetes. They were also older than the normal subjects. However, we have not observed a relationship between age and plasma H(e) concentrations in subjects aged 30 to 60 years. Nevertheless, further investigation is needed to determine the effect of age and various treatments for diabetes and their impact on the regulation of plasma H(e) concentrations by insulin. Our control population also was not matched with the diabetics in terms of other components of the insulin resistance syndrome such as blood pressure and plasma lipids. Further study is necessary to determine the impact of these factors on insulin's effect on plasma H(e). Furthermore, the possibility that the patients with diabetes had subclinical coronary artery disease also needs to be considered, as abnormalities of H(e) metabolism appear to be more prevalent in patients with vascular disease.

In a previous study, we found elevated H(e) levels in patients with NIDDM and not in patients with IDDM, suggesting that insulin resistance rather than hyperglycemia contributed to the elevated H(e) levels. These data are compatible with our previous observations and reports that hyperhomocystinemia only occurs in patients with NIDDM, not in patients with IDDM.[2]

Previous studies have suggested that H(e) is determined by the activity of two key enzymes, CBS, and MTHFR. The latter is the major determinant of H(e) in the fasting state, and CBS is the primary determinant of H(e) in the postprandial state. In the fasting state, plasma H(e) in diabetics was slightly lower than in nondiabetics, although the difference was not statistically significant. This makes it unlikely that MTHFR activity is abnormal in diabetes. Since insulin is secreted following an oral protein load, it is possible that insulin plays a role in decreasing postprandial H(e) levels by modulating CBS activity. If resistance to such an effect occurs, subjects with insulin resistance may develop post–methionine load hyperhomocystinemia as observed in NIDDM.[2]

It is well recognized that insulin decreases plasma methionine, the methionine being incorporated into newly synthesized protein.[13] Plasma amino acid concentrations, including methionine, decrease significantly following an oral glucose load and the subsequent increase in endogenous plasma insulin. However, in patients with diabetes, this decrease in amino acids does not occur, indicating a possible resistance to insulin's effect on amino acids in diabetics.[14]

The insulin-induced decrease in plasma methionine concentrations following insulin may be mediated through increased tissue uptake of methionine or metabolism via the transsulfuration pathway resulting in increased levels of H(e). Intracellular and plasma H(e) levels may then increase, particularly if a defect in CBS action exists.

The relative responsiveness of insulin to various amino acids may be modified by dietary intake, obesity, or starvation.[15] The patients and control subjects in our study were not on a standardized diet for prolonged periods, and vitamin B and folate intake may have been variable. This may explain the variation in the H(e) response to insulin.

The exact pathogenesis of the increased incidence of cardiovascular disease and the accelerated atherosclerosis in patients with NIDDM remains unclear. It has been suggested that it may be due to coinheritance of multiple cardiovascular risk factors.[5] Clustering of risk factors is seen in most patients with NIDDM, who also have insulin resistance, hypertension, dyslipidemia, hyperfibrinogenemia, increased platelet aggregation, and impaired fibrinolytic activity.[5] Hyperhomocysteinemia is also associated with a number of coagulation abnormalities and markers of endothelial dysfunction similar to those observed in patients with NIDDM and insulin resistance.[1]

H(e) metabolism is dependent on a number of vitamins that act as cofactors for the different enzymes in the metabolic pathways. Pyridoxine is an important cofactor in the activity of CBS, and its deficiency results in elevated H(e) levels following a methionine load. However, we have been unable to find a deficiency of pyridoxine, folate, or vitamin B_{12} in patients with NIDDM with hyperhomocysteinemia (unpublished observations, December 1997). A resistance to insulin action on CBS may be an alternative explanation for hyperhomocystinemia in these patients.

Recognition of hyperhomocysteinemia as a contributory factor in the development of accelerated vascular disease in patients with NIDDM is important, since plasma H(e) levels may be reduced with vitamin therapy. It is possible that some of the associated coagulation abnormalities may also be corrected,[1] leading to a reduction in the cardiovascular morbidity and mortality seen in NIDDM. Further investigation is warranted to clarify the role of insulin and insulin resistance in H(e) metabolism. The relationship between the insulin-induced decrease in plasma H(e) and other cardiovascular risk factors that cluster in the insulin resistance syndrome also needs to be investigated.

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