

The effect of glucose and insulin on the activity of methylene tetrahydrofolate reductase and cystathionine- β -synthase: studies in hepatocytes

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Abstract

Hyperhomocysteinemia is a well established risk factor for cardiovascular disease, and multiple factors likely lead to abnormal regulation of plasma homocysteine in patients with diabetes. To examine a possible role for insulin and glucose in homocysteine metabolism, we examined the activity of two important enzymes of homocysteine metabolism in hepatocytes. In various tissues of six mice, methylene tetrahydrofolate reductase (MTHFR) activity was present in all tissues tested and the highest concentration (per gram) was in the brain. In contrast, cystathionine β -synthase (CBS) activity appeared to be present only in the liver and to a small extent in the kidney. Using HEP G2 cells in culture, MTHFR activity was 3.3 ± 0.8 nmol/h when the glucose concentration in the medium was 100 mg/dl and fell to 2.3 ± 0.3 nmol/h when glucose was increased to 300 mg/dl. MTHFR activity was 3.4 ± 0.3 nmol/h when cells were exposed to an insulin concentration of 5 mU/ml and fell to 2.8 ± 0.3 nmol/h when insulin concentration was increased to 200 mU/ml ($P < 0.01$). In contrast CBS activity increased from 0.017 to 0.13 U/ml by increasing the glucose concentration in the medium ($P < 0.01$), but decreased from 0.04 to 0.02 ($P < 0.01$) when the insulin concentration was increased from 5 to 200 mU/ml, respectively. We conclude that CBS and MTHFR have different tissue distributions, with CBS being present predominantly in liver and kidney, and MTHFR found in many tissues. In addition, both insulin and glucose affect the activity of the two enzymes when added to hepatocytes in vitro. If such effects occur in humans with hyperglycemia and hyperinsulinemia, then alterations in homocysteine metabolism may contribute to the accelerated macrovascular disease associated with insulin resistance or type 2 diabetes. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Homocysteine is a sulfur-containing amino acid that is an intermediary in the metabolism of dietary methionine. Several enzymes play a role in methionine–homocysteine metabolism. Two of these enzymes, methylene tetrahydrofolate reductase (MTHFR) and cystathionine- β -synthase (CBS), are considered impor-

tant because mutations and dysfunction in these enzymes lead to clinically significant hyperhomocysteinemia [1]. Classic homocystinuria is due to a mutation in the CBS enzyme, resulting in severe hyperhomocysteinemia and premature atherosclerosis. It has recently been recognized that milder elevations in plasma homocysteine, usually due to mutations and/or acquired dysfunction of MTHFR or CBS, are an important risk factor for cardiovascular disease [1,2].

Previous studies have suggested that multiple factors control plasma homocysteine levels, including plasma levels of vitamins, such as folate and B12, and renal function [3–5]. Several lines of evidence have linked

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hyperhomocysteinemia to diabetes or insulin resistance. For example, we have previously demonstrated that diabetic patients with cardiovascular disease have elevated plasma homocysteine levels [6]. This was observed in insulin resistant type 2 diabetic subjects, and not in insulin deficient type 1 diabetic subjects, although type 1 diabetic subjects with nephropathy have elevated homocysteine levels [6,7]. Furthermore, acute hyperinsulinemia led to a fall in plasma homocysteine in normal subjects but not in insulin resistant patients with type 2 diabetes [8], suggesting that insulin lowers plasma homocysteine, and that type 2 diabetic subjects are resistant to this effect of insulin. Finally, rats fed a high fat, sucrose (HFS) diet developed obesity associated with hyperinsulinemia, insulin resistance [9], and hyperhomocysteinemia, along with changes in CBS and MTHFR [10].

Because we found abnormalities in homocysteine metabolism in type 2 diabetic humans, and in insulin resistant HFS rats, we further examined the role of glucose and insulin in homocysteine metabolism. The liver has generally been considered the most important organ in amino acid, including homocysteine, metabolism [11]. Hence, we studied the activity of MTHFR and CBS in cultured liver cells. After treating cultured liver cells with insulin and glucose, we determined that CBS and MTHFR were both decreased by high medium insulin, but responded in an opposite manner in response to glucose.

2. Methods

2.1. Animals

To examine homocysteine metabolism in mice, Six C57BL/6J mice (Jackson Labs, Bar Harbor, MA) at 8 weeks of age were maintained on a laboratory rodent diet (Harlan Sprague Dawley) containing standard mineral and vitamin mix for 4 weeks. Food and water were given ad lib on 12 h light and dark cycles. Mice were then fasted overnight, anesthetized with isoflurane, exsanguinated, and tissues removed for measurement of CBS and MTHFR activity.

2.2. Enzyme activity assays

2.2.1. Cystathionine β -synthase (CBS)

Tissues were immediately removed, washed free of blood, and frozen in liquid nitrogen until the assay was performed. CBS activity was assayed by modification of protocols by Mudd et al. [12] and Kraus [13]. Briefly, the tissues were homogenized in 0.05 M KH_2PO_4 (pH 7.5) and centrifuged at $10\,000 \times g$ at 4°C and the supernatant was collected. The enzyme reaction mixture contained: 0.1 M Tris (pH 8.6), 1 mM Pyridoxal Phos-

phate, 0.7 mg cellular protein extract, 0.5 mg/ml BSA, $0.06 \mu\text{Ci } ^{14}\text{C-Serine}$, 10 mM serine, 15 mM L-homocysteine, and 1 mM cystathionine. The reaction mixture was incubated for 3 h at 37°C , during which time the $^{14}\text{C-serine}$ was converted by CBS into $^{14}\text{C-cystathionine}$. The $^{14}\text{C-cystathionine}$ was separated from $^{14}\text{C-serine}$ by ascending paper chromatography using 2-propanol/formic acid/water (80:6:20) v/v as the mobile phase. The $^{14}\text{C-cystathionine}$ was cut out and counted in scintillation fluid. We defined 1 unit of enzyme activity as that which formed 1 nmol of cystathionine per h at 37°C . Enzyme activity was expressed as units/mg of cellular protein. Protein was determined using the BioRad protein reagent.

2.2.2. Methylene tetrahydrofolate reductase (MTHFR)

The MTHFR activity assay was modified from the procedure by Mudd et al. [14]. The tissues were homogenized in 0.25 M sucrose, centrifuged at $50\,000 \times g$ for 1 h and the supernatant was collected. The enzyme reaction mixture contained the following: 0.18 M KH_2PO_4 (pH 6.3), 3.6 mM menadione bisulfite, 1.4 mM EDTA, 7.2 mM ascorbic acid, 178 μM flavin adenine dinucleotide (FAD) and 420 μM of $^{14}\text{C-5-methyltetrahydrofolate}$. The reaction mix was incubated for 1 h at 37°C , and then terminated by the addition of 0.6 M sodium acetate pH 4.5, 0.1 M formaldehyde and 0.4 M 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 $^{14}\text{C-formaldehyde}$ was removed for counting in scintillation fluid. One unit of activity was defined as nmol of $^{14}\text{C-formaldehyde}$ formed per hour, and data were expressed per mg protein.

2.3. Cell culture

The HepG2 human hepatocyte cell line (human hepatocellular carcinoma, ATCC # HB 8065) was utilized. The cells were grown to confluency in DMEM containing 10% FCS in 100 mm tissue culture plates. The cells were then cultured in medium containing varying concentrations of glucose (100, 300 and 500 mg/dl) and insulin (5, 50 and 200 $\mu\text{unit/ml}$) for 4 days. When cells were in insulin, the glucose concentration was 100 mg/dl. These culture conditions, using glucose and insulin concentrations typical of those seen in diabetic humans, were chosen to best mimic the diabetic milieu in tissue culture. The 4-day culture conditions were intended to produce a chronic, rather than acute, effect on CBS and MTHFR. Cells were then harvested with 0.05% trypsin/EDTA, washed twice with phosphate-buffered saline (pH 7.4), centrifuged at $600 \times g$ for 5 min at 4°C , and stored at -70°C .

2.3.1. Statistics

Data were analyzed using a Student's *t*-test with significance reported at the level of $P < 0.05$. All data are expressed as mean \pm S.E.M.

3. Results

The results of the activities of CBS and MTHFR in various tissues of the mice are illustrated in Fig. 1. CBS enzyme activity was observed predominantly in the liver and kidney, with negligible activity in other tissues. In contrast, MTHFR activity was present in all tissues tested, and was highest in the brain, when expressed per milligram protein. Aortic tissue was available from two mice, and MTHFR activity was 1.74 U/mg protein, which was about the same as was found in heart and kidney, and CBS activity was essentially undetectable.

To determine whether elevated medium insulin or glucose affected CBS and MTHFR, studies were performed in vitro using Hep G2 cells. The effects of increasing the medium insulin concentration on Hep G2 cell MTHFR is shown in Table 1. An increase in the insulin concentration led to a stepwise and significant decrease in both CBS and MTHFR activity. The addition of insulin from 5 to 200 mU/ml decreased MTHFR activity from 3.4 to 2.8 U/mg, and decreased CBS from 0.04 to 0.02 U/mg.

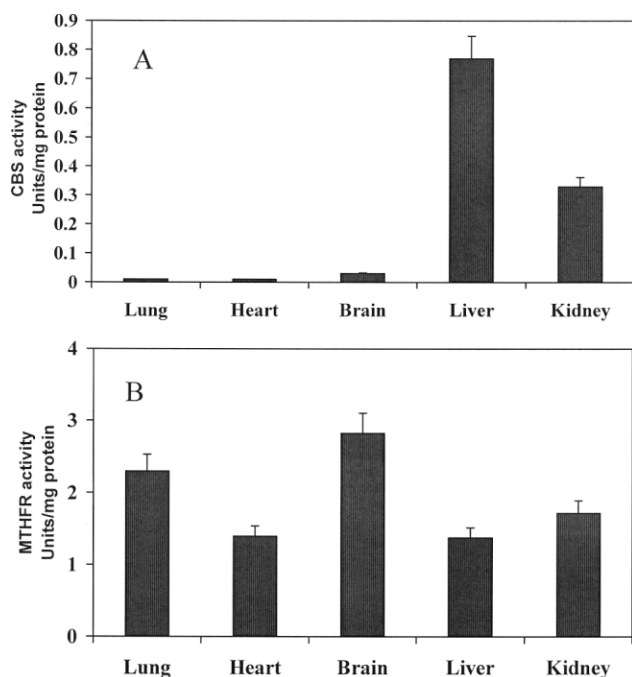


Fig. 1. (A) CBS enzyme activity in various tissues of mice; (B) MTHFR enzyme activity in mouse tissues. Data are expressed as Units per mg protein (see Section 2). The data are derived from six mice.

Table 1
Effect of insulin on CBS and MTHFR activity

Insulin (mU/ml)	MTHFR (U/mg)	CBS (U/mg)
5	3.4 \pm 0.27	0.04 \pm 0.003
50	3.0 \pm 0.15 ^b	0.03 \pm 0.003 ^a
200	2.8 \pm 0.29 ^a	0.02 \pm 0.004 ^a

^a $P < 0.01$ vs. insulin 5 mU/ml.

^b $P < 0.02$ vs. insulin 5 mU/ml; $n = 4-5$ for each point.

As shown in Table 2, increasing the glucose concentration of the medium from 100 to 300 mg/dl led to a significant and stepwise decrease in MTHFR activity from 3.3 to 2.3 U/mg with 300 mg/dl of glucose. In contrast, CBS activity changed in the opposite direction in response to increased medium glucose. CBS activity increased from 0.017 to 0.03 U/mg upon increasing the glucose concentration of the medium from 100 to 200 mg/dl, and there was no further increase with the higher glucose concentration. Thus, the addition of either insulin or glucose led to a decrease in MTHFR activity, whereas insulin and glucose had opposite effects on CBS.

4. Discussion

Many previous studies have linked elevated plasma homocysteine with arterial disease. In addition to many studies linking plasma homocysteine with atherosclerosis [3,15,16], other studies have demonstrated a relationship between hyperhomocysteinemia and arterial intimal-media thickness [17]. Recent large epidemiological studies have demonstrated that homocysteine is a significant risk factor for cardiovascular disease in patients with diabetes [18].

Patients with diabetes have an increased risk for atherosclerosis [19]. This increased risk is independent of concurrent conditions, such as dyslipidemia and hypertension, suggesting that there are additional factors in the diabetic milieu that contribute to vascular disease [20]. Most patients with diabetes have type 2 diabetes, which is characterized by insulin resistance. Recent studies have suggested that insulin resistance and hyperinsulinemia, even in the absence of diabetes, represents risk factors for atherosclerosis [21].

Table 2
Effect of glucose on CBS and MTHFR activity

Glucose (mg%)	MTHFR U/mg	CBS U/mg
100	3.3 \pm 0.8	0.017 \pm 0.003
200	2.8 \pm 0.5	0.032 \pm 0.003 ^a
300	2.3 \pm 0.3 ^b	0.030 \pm 0.004 ^a

^a $P < 0.01$ vs. glucose 100 mg/dl.

^b $P < 0.05$ vs. glucose 100 mg/dl; $n = 4-5$ for each point.

Several recent studies have suggested that patients with diabetes may have abnormalities in homocysteine metabolism. Diabetic patients with cardiovascular disease had elevated plasma homocysteine levels [6], and this was observed only in insulin resistant type 2 diabetic subjects, and not in insulin deficient type 1 diabetic subjects. Furthermore, acute hyperinsulinemia led to a fall in plasma homocysteine in normal subjects but not in insulin resistant patients with type 2 diabetes [8], suggesting that acute administration of insulin lowered plasma homocysteine, and that type 2 diabetic subjects were resistant to this effect of insulin. Thus, these studies suggest that the hyperinsulinemic insulin resistant environment of type 2 diabetes may alter plasma homocysteine levels.

Homocysteine is a thiol containing amino acid metabolized by remethylation to methionine or by transsulfuration to cysteine [1,11]. In the transsulfuration reaction, methionine is converted to homocysteine, which irreversibly condenses with serine to form cystathionine. This reaction is irreversible and is catalyzed by the pyridoxine dependent enzyme cystathionine β -synthase (CBS). Cystathionine is subsequently hydrolyzed to cysteine. In the remethylation pathway, methionine reforms from homocysteine when a methyl group is donated by *N*-5-methyltetrahydrofolate in a reaction catalyzed by the enzyme methylene tetrahydrofolate reductase (MTHFR). Alternatively, the methyl group may be donated by betaine. The remethylation pathway is the primary determinant of plasma homocysteine levels in the fasting state, while the transsulfuration pathway is most active following a methionine load.

In animal studies, an effect of both insulin deficiency and insulin resistance on enzymes in homocysteine metabolism has been described. In insulinopenic streptozotocin treated rats, insulin had an effect on hepatic cystathionine beta synthase (CBS) activity [22]. Plasma homocysteine fell in the diabetic rats and this fall was corrected by insulin. Insulin treatment led to a decrease in hepatic CBS activity in this model of type 1 diabetes. In rats fed a high fat, sucrose (HFS) diet, which caused mild obesity associated with hyperinsulinemia and insulin resistance [9], hyperhomocysteinemia was observed, along with decreases in CBS activity and increases in MTHFR activity [10]. Because abnormalities in homocysteine metabolism were found in situations of hyperinsulinemia and insulin resistance, we sought to better understand the regulation of homocysteine by examining the enzymes that are known to be involved in homocysteine metabolism *in vitro*.

As with most enzymes involved with amino acid metabolism, CBS and MTHFR are thought to be located predominantly in the liver [12], although there are little data on CBS and MTHFR enzyme activity in different tissues. Hence, we examined CBS and

MTHFR activity in different tissues in mice. Whereas CBS was predominantly found in the liver, MTHFR was found in many tissues, and indeed brain contained the highest amount of MTHFR when expressed per gram. These studies used the combined data from six mice. We are unaware of any previous studies that have examined MTHFR tissue distribution, and we are not aware of any previous description of the activity of the MTHFR enzyme in intact aortic tissue, although homocysteine metabolism is known to be active in endothelial cells [23]. Nevertheless, because of the large size of the liver when compared to other organs, these data support previous studies suggesting that the liver is a major organ in the expression of CBS and MTHFR activity.

To better understand the hepatic regulation of CBS and MTHFR, we cultured Hep G2 cells, and examined the response to insulin and glucose. After treating Hep G2 cells with insulin and glucose, we determined that CBS and MTHFR were both decreased by high medium insulin, but responded in an opposite manner in response to glucose. By increasing glucose concentration in the medium, Hep G2 cells demonstrated lower MTHFR activity, but higher CBS activity.

These data may suggest that homocysteine metabolism is disordered in subjects with insulin resistance and type 2 diabetes, and may be modulated by glucose and insulin. To the extent that these data can be extrapolated to humans, these data would suggest that insulin resistance and hyperinsulinemia would be particularly detrimental. Hyperinsulinemia would lower the levels of both CBS and MTHFR. Elevated glucose, as in a diabetic patient, would variably elevate CBS, but the hyperinsulinemic environment of the insulin resistant non-diabetic subject would not demonstrate this compensation by CBS, resulting in lower levels of both enzymes. Hence, one might predict that plasma homocysteine levels in type 2 diabetic patients would be variable, since insulin and glucose levels in such patients are variable. However, patients with insulin resistance and hyperinsulinemia in the absence of diabetes would be more likely to demonstrate low levels of CBS and MTHFR. Although few clinical studies have examined this, a recent cross-sectional study of non-diabetic subjects demonstrated a significant negative correlation between plasma homocysteine and fasting insulin [24].

We conclude the homocysteine metabolizing enzymes CBS and MTHFR have different levels of activity in various tissues of the mouse. In addition, both insulin and glucose affect the activity of these enzymes when added to hepatocytes *in vitro*. The relative importance of transsulfuration and remethylation in homocysteine metabolism is complex and poorly understood, and many factors such as diet, obesity, and insulin resistance likely play a role that requires further investigation. Further investigation is required to determine the

exact mechanism and importance of glucose, insulin and insulin resistance in the pathogenesis of hyperhomocysteinemia.

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