Cystathionine-β-Synthase Deficiency: Detection of Heterozygotes by the Ratios of Homocysteine to Cysteine and Folate

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Elevated total plasma homocysteine (tHcy) is recognized as an independent risk factor for occlusive vascular disease. However, it is not known how much of the observed hyperhomocysteinemia in patients with vascular disease is due to heterozygosity for cystathionine-β-synthase (CβS) deficiency, because a clinically useful screening method is unavailable. To determine this, parents of children who are homozygous for CβS deficiency (affected with homocystinuria) and a control population were compared for tHcy, total plasma cysteine (tCys), plasma folate, and plasma vitamin B_{12}. The group of obligate heterozygotes had increased tHcy ($P \leq .01$), decreased tCys ($P \leq .01$), and decreased plasma folate ($P \leq .01$). The calculated ratios of tHcy/tCys ($P = .01$) and tHcy/plasma folate ($P = .003$) were the best metabolic discriminants for genotype. These ratios are likely to prove useful in heterozygote screening for CβS deficiency and in the development of rational treatment strategies for patients with increased tHcy.

THE DETECTION OF HETEROZYGOTES for homocystinuria is of considerable clinical importance because of the association with elevations of total plasma homocysteine (tHcy) and vascular disease.[1-4] Homocysteine is an amino acid intermediate of methionine metabolism (Fig 1), with two possible metabolic fates. Homocysteine can be remethylated to form methionine by a pathway requiring methyl transfer from N^5-methyltetrahydrofolate to vitamin B_{12}, forming methyl B_{12}, and subsequent transfer by N^5-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase, EC 2.1.1.13) to homocysteine, forming methionine. Alternatively, homocysteine can undergo transsulfuration. This is initially catalyzed by cystathionine-β-synthase ([CβS] EC 4.2.1.22) in a reaction requiring pyridoxal phosphate as a cofactor and serine as a substrate. Cysteine, α-ketobutyrate, and ammonia (NH_3) are the products of cystathionine cleavage by the enzyme cystathionine-γ-lyase (EC 4.4.1.1).[5]

Impaired CβS activity causes the most common autosomal-recessive form of homocystinuria.[6] Parents of persons affected with homocystinuria are obligate heterozygotes for CβS deficiency. Persons affected with homocystinuria produce large amounts of homocystine, the oxidized form of homocysteine, in the plasma and urine. Untreated individuals with this disorder usually develop premature occlusive vascular disease, osteoporosis, dislocated optic lenses, and developmental delays.[6] The carrier frequency for CβS deficiency estimated from the rate of detection of homozygotes during screening of the newborn population is approximately one in 100 (1%).[6] Previous attempts to identify obligate heterozygotes for homocystinuria have been unsuccessful. Despite strategies to amplify the metabolic differences of carriers (for CβS deficiency) by obtaining samples from both fasting and methionine-challenged subjects, tHcy concentrations observed in carriers have always overlapped those observed in controls.[6] Other methods to detect carriers for homocystinuria have included measurement of CβS enzyme activity in several cell types. However, these measurements have also been unable to discriminate between carriers and controls.[6]

The inability to detect heterozygotes for a recessive metabolic trait is not an unusual problem when only the substrate in a presumably blocked reaction is used as a metabolic discriminant. Because the ratios of substrate and product of an impaired reaction were useful for the detection of heterozygotes for phenylketonuria,[7,11,12] we tested the hypothesis that certain ratios of substrates, cofactors, and products in homocysteine metabolism can distinguish heterozygotes from a control population. In this study, tHcy, total plasma cysteine (tCys), plasma folate, and plasma vitamin B_{12} were quantified and their ratios used as metabolic discriminants for the genotyping of heterozygotes for CβS deficiency.

SUBJECTS AND METHODS

Subjects

Seven obligate heterozygotes were the parents of probands with classic homocystinuria, and included five mothers and two fathers aged 28 to 47 years. Samples were either fasting or semifasting (no food for at least 3 hours) and were collected at the affected child's clinic visit. The comparison group included 96 women aged 50 to 79 years. All of these samples were obtained under fasting or semifasting conditions. This study was approved by the Emory University Human Investigation Committee.

For both groups, the blood sample was anticoagulated in EDTA, chilled on ice for 30 minutes, and centrifuged. The resulting plasma was divided between two vials and frozen. Plasma collected for folate and vitamin B_{12} determination was stored at −70°C until analyzed. Plasma collected for tHcy determination was stored at −20°C until analyzed. All analytes quantified were stable for at least 12 months under these conditions, and all analyses were completed within 9 months of sample collection.

Analytical Methods

tHcy concentrations were measured using high-performance liquid chromatography with fluorescence detection using the method of Araki and Sako,[8] with modified sample preparation as follows. *N*-acetyl-L-cysteine was used as an internal standard for derivatization and injection volume accuracy, with 50 μL 0.25-mmol/L solution added to 200 μL plasma. All disulfide bonds were reduced by adding 25 μL 10% tri-*n*-butylphosphine in dimethyl formamide to the plasma with internal

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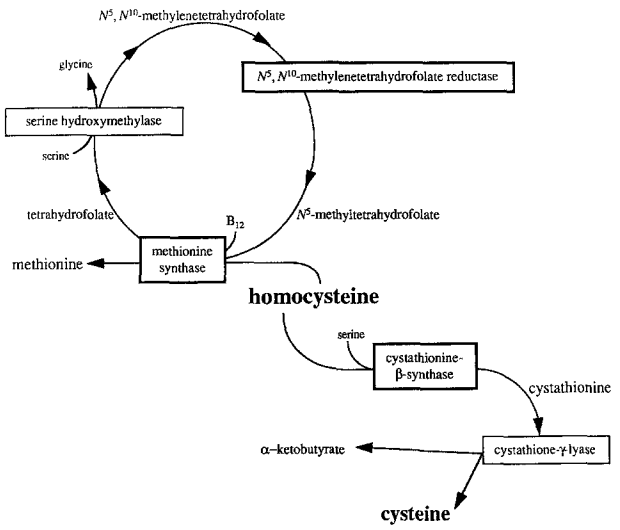

Fig 1. Remethylation and transsulfuration of homocysteine. Remethylation: The roles of folate and vitamin B_{12} are depicted for clarity. Folate, as N^5-methyltetrahydrofolate, is formed from N^5, N^{10}-methylenetetrahydrofolate by MTHFR, before transfer of the methyl group to cobalamin, forming methyl B_{12}. The enzyme methionine synthase then transfers this methyl group to homocysteine, forming methionine.[5] Theoretically, inherited blocks at either enzyme or a deficiency of either vitamin precursor can cause hyperhomocysteinemia. Transsulfuration: The transsulfuration reactions of homocysteine eventually form cysteine. These reactions require that CβS, with pyridoxal phosphate as a cofactor, condense homocysteine and serine to form cystathionine. Cleavage of cystathionine by cystathione-γ-lyase yields cysteine, α-ketobutyrate, and NH_3.[5] Impaired CβS activity causes an autosomal recessive form of homocystinuria, resulting in elevated tHcy and decreased tCys.[6] Other aspects of homocysteine metabolism are not illustrated. These other reactions include the demethylation of methionine to homocysteine through an adenosyl derivative, and the liver-specific remethylation of homocysteine to methionine using betaine as a methyl donor. Complete pathways for homocysteine metabolism illustrate these reactions.[5,6]

standard. After incubation at 4°C for 30 minutes, sample proteins were precipitated using 155 μL 0.6-mol/L perchloric acid with 1 mmol/L EDTA. The sample was then mixed using a vortex-type mixer and incubated 10 minutes at room temperature before centrifugation. A 200-μL aliquot of supernatant was treated with 200 μL 10% 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid in 100 mmol/L potassium borate with 2 mmol/L EDTA, pH 9.5, and 400 μL saturated potassium borate with 5 mmol/L EDTA, pH 10.5. The mixture was incubated for 30 minutes at 60°C using a dry-heat block and then cooled by ambient air for 15 minutes to room temperature. The injection volume was 50 μL for Hcy and 25 μL for tCys determination. Separation and detection were essentially performed as previously described.[8] All samples were assayed in triplicate. The tCys determination was made separately in this study on samples from five carriers and 39 controls with sufficient volume for this additional assay.

Plasma folate and plasma vitamin B_{12} were determined simultaneously in the same aliquot of EDTA plasma using a radioligand-binding assay (Quantaphase II B_{12}-Folate Radioassay; BioRad, Hercules, CA). Concentrations of folate and vitamin B_{12} were determined by comparison to a six-point standard curve. Three commercially available serum pools for both vitamins were tested as samples to verify that separte assays were equivalent (BioRad Lyphochek Immunoassay Control-Trilevel). All samples were assayed in duplicate.

Statistical Analyses

Median values and nonparametric tests were used to compare these groups because of the skewed nature of the biological data. In addition, the number of subjects in each group was vastly different. All P values comparing median concentrations were derived using the nonparametric Kruskal-Wallis test. P value less than or equal to .05 was considered significant. Confidence intervals around median values were calculated using the method of Gardner and Altman.[9] A 95% confidence interval around a median is equivalent to the range of values when there are fewer than nine observations.[9]

Calculations were made for sensitivity and specificity using the formulas,[10] sensitivity = a/(a + c) and specificity = d/(b + d), where a represents those who test positive and are known carriers (true positive); b, those who test positive and are not known carriers; c, those who test negative and are known carriers (false negative); and d, those who test negative and are not known carriers.

RESULTS

The distributions of tHcy, tCys, and plasma folate concentrations in the obligate carrier and control groups were statistically different. Distribution data are summarized in Table 1, and include the mean ± SD, median, 95% confidence interval around the median, and statistical differences between the distributions of analyte concentrations in the experimental groups. In addition, the number of individuals who provided samples for each of the analytes are noted on Table 1. Obligate heterozygotes for homocystinuria had higher concentrations of tHcy ($P = .01$) and lower concentrations of both tCys ($P \leq .02$) and plasma folate ($P < .01$) than the control group. The distribution of plasma vitamin B_{12} concentrations was not significantly different ($P > .05$) between the carriers and controls. The overlap observed in the range of concentrations for tHcy in both obligate heterozygotes and controls is consistent

Table 1. Concentrations of the Plasma Analytes tHcy, tCys, Folate, and Vitamin B_{12} in Obligate Heterozygotes for CβS Deficiency and in a Comparison Population

	Obligate Heterozygotes			Comparison Population			
Plasma Analyte	No. of Subjects	Mean ± SD (range)	Median (95% confidence interval)	No. of Subjects	Mean ± SD (range)	Median (95% confidence interval)	P†
tHcy (μmol/L)	7	16.7 ± 8.8 (8.7-31.4)	13.9 (8.7-31.4)	96	9.8 ± 2.4 (5.5-19.0)	9.7 (9.2-10.4)	.01*
tCys (μmol/L)	5	260 ± 54 (164-294)	286 (164-294)	39	333 ± 64 (256-359)	314 (297-349)	.02*
Folate (ng/mL)	7	3.5 ± 3.2 (1.2-9.0)	1.9 (1.2-9.0)	96	7.7 ± 5.5 (2.2-31.2)	5.7 (5.2-7.3)	.01*
Vitamin B_{12} (pg/mL)	7	368 ± 143 (140-565)	410 (140-565)	96	518 ± 252 (37-1,298)	479 (420-588)	.12

NOTE. The 95% confidence interval around the median is equivalent to the range of values for the small sample size in the obligate heterozygote group.

*Statistically significant if $P \leq .05$.

†Kruskal-Wallis test.

with previous reports.[1,6] There is also overlap observed for tCys, plasma folate, and plasma vitamin B_{12} levels between these groups. This overlap of ranges obviated the use of the concentration of individual analytes as a metabolic discriminant for heterozygosity of CβS deficiency.

A comparison of the ratios of biological components that differ between the two groups is summarized in Table 2, with the median and 95% confidence interval around the median. The ratios of both tHcy/tCys ($P = .01$) and tHcy/folate ($P = .003$) are higher in the obligate heterozygote group than in the comparison group. Although individual metabolite concentrations could not discriminate between carriers and controls, the ratios of tHcy/tCys and tHcy/folate provided nearly complete discrimination.

Sensitivity and specificity are accepted parameters used to evaluate the accuracy and precision of a screening test.[10] For CβS carrier screening, sensitivity is the ability of the test to correctly identify known carriers for CβS deficiency, and specificity is the ability of the test to group the control population as not carrying CβS deficiency. Sensitivities and specificities were calculated to estimate the ability of both the individual analytes and the ratios to correctly identify carriers and controls. The following thresholds were chosen to provide optimal separation of the two comparison groups and to define limits for classification as nonheterozygous for CβS deficiency. These thresholds are as follows: tHcy, 12.5 μmol/L or less; tCy, 290 μmol/L or greater; plasma folate, 3 ng/mL or greater; tHcy/tCys, 4.30×10^2 or less; and tHcy/plasma folate, 4.40 or less. Table 3 summarizes the sensitivity and specificity of the individual plasma analytes and ratios. A "corrected population" was created by excluding a participant with a known confounding variable. This parent of an affected child, who tested negative by both ratios, has been taking many vitamins for several years, including a complete multivitamin containing 2 mg vitamin B_6, 400 μg folate, and 9 μg vitamin B_{12}, in addition to individual supplements of B-complex, vitamin C, and vitamin E. Table 3 displays these recalculated sensitivities and specificities for analytes and their ratios omitting this individual. The sensitivity of tHcy, plasma folate, tHcy/tCys, and tHcy/folate increased in the corrected population.

DISCUSSION

Previous attempts to provide metabolic discrimination of CβS carriers have failed. The ratios of analytes shown herein provide the first useful metabolic discriminant for heterozygous CβS deficiency. These data show that tHcy, tCys, and plasma folate concentrations are significantly different between the carriers and the control group, and the plasma vitamin B_{12} concentration is not different between these groups. The ratios of tHcy/tCys and tHcy/folate provide the best prediction of genotype. These preliminary results suggest that the ratios of tHcy/tCys and tHcy/folate could be used to screen individuals with hyperhomocysteinemia of unknown etiology to ascertain whether the mechanism is heterozygosity for CβS deficiency.

Table 2. Ratios of tHcy, tCys, and Folate in Obligate Heterozygotes for CβS Deficiency and in a Comparison Population

	Obligate Heterozygotes		Comparison Population		
Ratio of Plasma Analytes*	Median	95% Confidence Interval	Median	95% Confidence Interval	*P*
tHcy/tCys	5.3	3.1-9.3	3.1	2.9-3.6	.01†
tHcy/folate	7.3	1.0-20.9	1.5	1.4-2.0	.003†

NOTE. The 95% confidence interval around the median is equivalent to the range of values for the small sample size in the obligate heterozygote group.

*Ratio multiplied by 10^2.

†Statistically significant at $P \leq .01$ using the Kruskal-Wallis test.

Table 3. Sensitivity and Specificity of Individual Plasma Analytes and Ratios in Obligate Carriers of CβS Deficiency and in a Comparison Population

Parameter	Threshold Not a Carrier for CβS Deficiency if	All Experimental Subjects		Corrected Population†	
		Sensitivity	Specificity	Sensitivity	Specificity
tHcy	≤12.5 μmol/L	71.4%	91.7%	83.3%	91.7%
tCys	≥290 μmol/L	80.0%	74.4%	75.0%	74.4%
Folate	≥3.0 ng/mL	71.4%	90.6%	83.3%	90.6%
tHcy/tCys*	≤4.30	80.0%	92.3%	100.0%	92.3%
tHcy/folate	≤4.40	71.4%	96.9%	83.3%	96.9%

*Ratio multiplied by 10^2.

†One known carrier omitted due to documented megavitamin supplementation.

A high tHcy/tCys was postulated to be able to define impaired CβS activity, since cysteine is an ultimate product of homocysteine transsulfuration (Fig 1) and is decreased in the plasma of homozygous affected patients.[6] From previous studies in our laboratory for heterozygote detection in phenylketonuria, we used a substrate to product ratio and found that the ratio of phenylalanine to tyrosine discriminated heterozygotes from controls for impaired phenylalanine hydroxylase.[7,11,12] In this investigation, we did find that obligate heterozygotes for CβS deficiency have a significantly higher tHcy/tCys ratio than the control group.

The etiology of a high tHcy/folate ratio may be genetic, environmental, or both. Genetic etiologies include "slow" mutations in folate metabolism. One common mutation is a C to T transition in base pair 677 of the N^5,N^{10}-methylenetetrahydrofolate reductase ([MTHFR] EC 1.1.1.171; Fig 1) gene that substitutes a valine for an alanine at amino acid 175.[13] Homozygosity for this mutation occurs in 5% to 10% of the population of Europe and Canada, and has been associated with increased tHcy in the presence of low plasma folate.[13] This defect might impair homocysteine remethylation to methionine by retarding the folate cycle and decreasing the rate of production of N^5-methyltetrahydrofolate, the primary form of extracellular (plasma) folate. However, one would expect this to increase the concentration of tCys by increasing the substrate concentration for CβS and, consequently, the transsulfuration pathway. A high tHcy/plasma folate ratio might also be a reflection of the nutritional status of the individual. Dietary histories were not collected as part of this preliminary investigation, but would have been useful to approximate the folate present in the diet of all participants. Low plasma folate from inadequate intake could be confirmed by evaluation of erythrocyte folate. This intracellular indicator of tissue folate status was not assessed in this investigation.

The low plasma folate found in the carrier population was unexpected. It is established that patients with homocystinuria have an increased requirement for folate.[14-16] Could obligate heterozygotes also have an increased requirement for folate? This theoretical need could stem from a genetic retardation of transsulfuration, resulting in increased homocysteine concentrations, active remethylation to methionine, and increased demand for all available N^5-methyltetrahydrofolate. Perhaps the folate cycle is not able to produce adequate N^5-methyltetrahydrofolate to meet the extra remethylation requirement.

Vitamin B_{12} deficiency causes hyperhomocysteinemia by functional impairment of the B_{12}-dependent enzyme methionine synthase (Fig 1), thus impeding homocysteine remethylation to methionine. Therefore, one would predict that in vitamin B_{12} deficiency or dependency states, plasma methionine would be low in the presence of elevated tHcy. However, tCys concentrations would not be reduced due to increased substrate, as previously described, resulting in increased activity of the transsulfuration pathway. In this investigation, the distribution of plasma vitamin B_{12} concentrations was not different between the groups, although both groups contained a few individuals with plasma vitamin B_{12} less than 200 pg/mL. For this reason, it is unlikely that differences in the ratios of tHcy/tCys and tHcy/folate were caused by vitamin B_{12} deficiency.

Several possible limitations exist in this investigation. It is possible that the presumed fathers may not be the biological parent of the affected child. It is likely that the control group contains heterozygotes for CβS deficiency. Based on the estimated carrier rate of 1%, one individual in the comparison population could be expected to be an undiagnosed carrier for CβS deficiency, and this would account for a high tHcy in the group presumed to be homozygous normal for CβS deficiency. Because there is no practical mechanism to genotype the CβS gene in the general population, we did not delete any observations in the control group.

This group of parents included several different mutations in the gene for CβS (Kruger WD, personal communication, April 1997), producing a wide range of impaired function. Some are parents of children with vitamin B_6–responsive homocystinuria, and at least one parent who overlapped with our control population was consuming supraphysiological amounts of vitamins. An unknown number of control individuals were probably taking vitamins, as evidenced by a few very high concentrations of plasma folate and vitamin B_{12}. Because of outlier data for both controls and obligate heterozygotes, distributions rather than means were compared.

The control population was not age- and gender-matched to the carrier population in this investigation. The comparison population is all female and older than the obligate heterozygote group. Others have observed that tHcy concentrations increase with age, and that this finding might be a combination of the aging process and the high prevalence of inadequate vitamin B_{12}, folate, and vitamin B_6 status in healthy elderly persons.[17] The use of this comparison population, who were older than the obligate heterozygotes for CβS deficiency, was a bias against our finding of lower plasma folate and higher tHcy concentrations in the younger carrier group.

An additional limitation is the possibility of MTHFR defects in both controls and carriers that could affect the concentration of both tHcy and plasma folate in each group. Homozygosity for the common C677T mutation of MTHFR was observed in 73.1% of the participants in the Hordaland Homocysteine Study, who had tHcy concentrations of 40 μmol/L or higher in nonfasting samples.[18] These hyperhomocysteinemic subjects were also reported to have lower plasma folate (median, 1.3 ng/mL), lower vitamin B_{12}, and lower vitamin intake, to consume more coffee, and to smoke more frequently than participants without hyperhomocysteinemia.[18] No genetic analysis was performed for CβS deficiency, but one subject identified in the hyperhomocysteinemia group was referred for treatment of homocystinuria and was therefore removed from this investigation.[18] Another study identifying individuals homozygous for the MTHFR C677T mutation observed that persons with a plasma folate concentration less than the median for the study population (6.8 ng/mL) had greater elevations of tHcy than homozygotes with a plasma folate concentration greater than the median.[19] These investigations suggest that homozygous C677T MTHFR is associated with hyperhomocysteinemia in individuals with a plasma folate concentration less than the median of the distribution of observations. The common C677T MTHFR mutation may be present in individuals included in the present investigation. This could be a bias against the specificity of plasma tHcy, folate, and the tHcy/folate ratio to identify carriers for CβS deficiency. The use of both ratios, tHcy/folate and tHcy/tCys, would theoretically be more informative for the presence of CβS deficiency.

There are both positive and negative answers to the hypothesis that heterozygotes for CβS deficiency have an increased risk for premature vascular disease. In some studies, there was evidence against this hypothesis. In a sample of four persons with premature occlusive arterial disease, no genetic changes were observed in mRNA for CβS between the case group and normal "wild-type" mRNA.[20] A guanine to adenine transition at nucleotide 919 of the CβS gene results in a serine replacing glycine at amino acid 307 (G307S) of the CβS protein. Homozygotes for the G307S mutation are severely affected and are not responsive to pyridoxine.[21] Heterozygotes for this mutation exhibit hyperhomocysteinemia.[22] Since the relative allele frequency of G3075 among Irish patients with CβS deficiency is 71% and one in 49,000 newborns have homocystinuria in Ireland,[23] approximately one in 157 Irish may be carriers for G307S. This mutation was not detected in 111 Irish patients with premature coronary heart disease, but it was present in two of 105 controls.[24] However, the power to detect a difference in the frequency of G307S in these small populations was only 3.5%. This Irish study did observe that individuals homozygous for the C677T MTHFR have a higher risk of premature coronary heart disease,[24] but this was not the case with US physicians.[25] An investigation of both CβS and MTHFR concluded that heterozygous CβS deficiency was not involved in premature vascular disease, whereas C677T MTHFR homozygosity was a risk factor.[26] However, this investigation screened 60 cardiovascular patients for a mutation identified to occur in only 50% of those homozygous for CβS deficiency in the reference (Dutch) population.[26] With 33 reported mutations identified in CβS,[20] screening for a single mutation to identify heterozygous CβS deficiency in an uncharacterized population would not be definitive. Numerous investigations have reported

hyperhomocysteinemia in obligate heterozygotes for CβS deficiency.[6,21,22] This study also reports the presence of hyperhomocysteinemia in the majority of obligate heterozygotes for CβS deficiency who participated. Because a dose-response effect has been reported between tHcy concentrations and the risk for vascular disease,[27] further studies are required of the frequency of CβS heterozygotes in populations with premature vascular disease. The ratios of tHcy/folate and tHcy/tCys developed here for the identification of heterozygotes for several different mutations producing CβS deficiency will be useful for this endeavor.

We conclude that heterozygotes for several different mutations producing CβS deficiency are identifiable by quantifying sulfur amino acids and the precursor vitamins of active cofactors involved in a blocked catalytic reaction. The ratios of these analytes are better discriminants than the individual concentrations. The ratios of tHcy/tCys and tHcy/folate identify heterozygotes for CβS deficiency.

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