Plasma homocysteine concentration does not reflect intracellular liver homocysteine in rats.

Desirée E.C. Smith¹, Yvo M. Smulders²,³, An S. De Vriese⁴, Henk J. Blom¹,³

¹ Department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands. ² Department of Internal Medicine, VU University Medical Center, Amsterdam, The Netherlands. ³ Institute for Cardiovascular Research, ICaR-VU, VU University Medical Center, Amsterdam, The Netherlands. ⁴ Renal Unit, Department of Internal Medicine, AZ Sint-Jan Brugge-Oostende AV, Brugge, Belgium

Submitted
Abstract

Objective
Elevated plasma homocysteine (Hcy) concentrations have been correlated to a higher incidence of a wide range of disorders such as neural tube defects and cardiovascular disease. Whether plasma Hcy concentration (and changes therein) are an accurate reflection of intracellular concentrations (and changes) in organs like the liver is unknown.

Methods
Wistar rats were fed Hcy-increasing diets (B-vitamin depletion, high-methionine) for 8 weeks. Plasma and liver concentrations of one-carbon metabolism metabolites, and activity of cystathionine ß-synthase and methylenetetrahydrofolate reductase were measured by LC-MS/MS.

Results
Plasma and liver Hcy and 5-methyltetrahydrofolate concentrations did not correlate (R=0.60, p=0.09;R=0.18, p=0.63, respectively). Liver Hcy correlated with liver S-adenosylmethionine (R=0.72, p=0.03), but not with liver 5-methyltetrahydrofolate and S-adenosylhomocysteine. The B-vitamin depleted diet increased plasma Hcy (p=0.00) but decreased liver Hcy (p=0.00). The high-methionine diet increased plasma Hcy, but did not alter liver Hcy, S-adenosylmethionine or S-adenosylhomocysteine. The high-methionine diet resulted in an increase in cystathionine ß-synthase and methylenetetrahydrofolate reductase activity. The B-vitamin depleted diet had no effect on the activity of these 2 enzymes.

Conclusions
Plasma one-carbon metabolite concentrations are not a reflection of liver concentrations. Hcy-increasing diets show several unexpected effects of liver one-carbon components, indicating that one-carbon metabolism is more complex than meets the eye when just looking at plasma concentrations.
Introduction

The amino acid homocysteine (Hcy) is part of the tightly regulated one-carbon (1C) metabolism (Figure 1).\(^1\) Elevated concentrations of plasma Hcy have been the focus of many studies since moderately elevated concentrations were shown to predict cardiovascular disease (CVD), as well as several other disorders.\(^2\)\(^-\)\(^4\) Exactly what causes plasma Hcy concentrations to become elevated, and whether plasma Hcy itself is toxic or a merely a biomarker of disrupted one-carbon metabolism, remains unclear. To date, several determinants of plasma Hcy have been identified, including age, sex, dietary factors (B-vitamin status, methionine intake), and genetic factors (mainly the methylenetetrahydrofolate reductase (MTHFR) 677C>T polymorphism).\(^5\)

![Figure 1. Simplified scheme of one-carbon metabolism.](image)

SAM (S-adenosylmethionine), SAH (S-adenosylhomocysteine), THF (tetrahydrofolate), MTHFR (methylenetetrahydrofolate reductase), CBS (cystathionine β-synthase), MAT (methionine adenosyl transferase), MS (methionine synthase).

In order to elucidate the meaning of elevated plasma Hcy concentrations, it is important to understand where plasma Hcy originates from. Most likely, many tissues contribute to the plasma Hcy equilibrium (the sum of export and uptake of cells/organs).\(^6\) Since liver, kidney and pancreas are the only organs with an active transsulfuration pathway (irreversible conversion to cystathionine (Cysta)), these organs probably play a leading role in the
metabolization of Hcy. However, the kidney does not significantly filter or convert Hcy. This leaves the liver as the plausible major contributor to Hcy conversion, and therefore plasma Hcy concentrations might be expected to mirror hepatic concentrations. While liver in particular may be responsible for influencing concentrations of Hcy in plasma, plasma Hcy concentrations in turn could contribute to intracellular Hcy concentrations of different cell types and organs. Elevated concentrations of plasma Hcy have for example been shown to increase influx of Hcy into the heart.

There are studies that have investigated Hcy or other 1C metabolites in tissues, however the correlation between intracellular Hcy and plasma Hcy in particular has been widely understudied. As a result, our knowledge of intracellular 1C metabolite concentrations in hyperhomocysteinemic states is incomplete.

This study focuses on examining the correlation between plasma and liver Hcy concentrations, and between liver Hcy and other 1C metabolites in rats. Apart from assessing these correlations in static conditions, we studied the effects of diet induced hyperhomocysteinemia on 1C metabolites in liver and plasma, and on cystathionine β-synthase (CBS) activity and MTHFR activity in liver.

Materials and methods

Study population
At the age of 4 weeks female Wistar rats were divided in 3 groups. Group 1 (VD, n=8) was fed a diet with low concentrations of folate, vitamin B6, and vitamin B12. Group 2 (HM, n=8) was fed a diet rich in methionine (Met). Group 3 (control, n=10) was fed a standard rodent chow (Harlan Teklad, Indianapolis, United States of America). All diets were matched for kilocalories, and the rats were allowed free access to food and water. The rats were sacrificed after 8 weeks on the diet. Tissues were extracted, frozen in liquid nitrogen and stored at -80°C for 10 years. Met rich diets contained 7.7 g/kg Met, were the normal Met diets contained 3.8 g/kg Met. Vitamin B deficient diets contained only residual amounts of folate, vitamin B6 and vitamin B12, whereas normal diets contained 2 mg/kg folic acid, 30 µg/kg vitamin B12 and 70 mg/kg pyridoxine. Animals received care in accordance with national guidelines for animal protection.

Sample preparation
Frozen liver was homogenised using Potter-Elvehjem tubes at 4°C. For the determination of folates, CBS activity and MTHFR activity, approximately 10
mg of liver was homogenised in 50 mM phosphate buffer (PH=7) on ice. For the determination of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) 50 mM isocitrate buffer (pH=4) was used. For the determination of Hcy, Met and Cysta 50 mM phosphate buffer (pH=7) containing 50 mM dithiothreitol was used. Homogenates were centrifuged (10,000 g, 10 min, 4ºC), and the supernatant was used for the metabolite/activity determinations.

Procedures
Hcy and Cysta in plasma and Hcy, Met and Cysta in liver extracts were measured by LC-MS/MS (API3000, Applied Biosystems, Foster City, CA, USA). Intra-assay CVs (n=6) were <9% for Hcy, Met & Cysta.\textsuperscript{15} SAM and SAH in liver extracts were measured with LC-MS/MS (API3000, Applied Biosystems, Foster City, CA, USA). Intra- and inter-assay CVs for SAM were 6.8% and 4.2%, respectively. The intra- and inter-assay CVs for SAH were 6.9% and 5.5%, respectively.\textsuperscript{16} Folate fractions in plasma and liver extracts were measured with LC-MS/MS (API3000, Applied Biosystems, Foster City, CA, USA). Intra- and inter-assay CVs for 5-methyltetrahydrofolate (5MTHF) were 1.2% and 2.8%, respectively. Intra- and inter-assay CVs for non-methylTHF as a group were 1.6% and 1.5%, respectively. Intra- and inter-assay CVs for FA were 3.2% and 7.1%, respectively.\textsuperscript{17} CBS activity in liver extracts was measured by incubating liver extracts in 90 mM Tris buffer (pH=8.6) containing 4.5 mmol/L EDTA, 2.7 mmol/L Hcy and 7.3 mmol/L serine for 4 hours at 37ºC, and measuring the produced cystathionine with LC-MS/MS (API3000, Applied Biosystems, Foster City, CA, USA). Intra- and inter-assay CVs for CBS activity were 5.2% and 14.7%, respectively.\textsuperscript{18} MTHFR activity in liver extracts was measured by incubating liver extract in 250 mmol/L Tris buffer (pH=6.5) containing 2.5 mmol/L EDTA, 2.5 µmol/L R-5,10-methenylTHF and 1 mmol/L NADPH for 30 min at 37ºC. The amount of 5MTHF formed was measured by LC-MS/MS.\textsuperscript{17} Intra- and inter-assay variations were 2.6% and 12.1%, respectively.

Statistics
SPSS 17.0 was used for all statistical calculations. First, the correlation (Spearman rank) between liver and plasma Hcy concentrations was
determined. Subsequently, the correlation between liver Hcy and selected liver 1C metabolites was determined (SAM, SAH, 5MTHF). This selection of correlations to address was based on established plasma correlations (5MTHF and SAH) and rationale (SAM).

In order to establish whether the different diets caused an altered distribution of 1C metabolites in liver and plasma, and of enzyme activity in liver, differences between these parameters were analysed using one-way Anova.

**Figure 2.** Effect of diet on plasma and liver Hcy concentrations.

**Difference is significant at the 0.01 level (2-tailed). HM (high methionine diet), VD (vitamin deficient diet).

**Results**

**Stability of the measured metabolites**

Since samples that were stored for 10 years were used, first the stability of all metabolites was investigated during prolonged storage using studies that were measured before. In plasma Met, SAM and SAH proved to be unstable and were therefore omitted from the results. Intracellular, all metabolites proved stable during storage at -80ºC.
Correlations between plasma and liver concentrations of Hcy and 5MTHF
In the control group, plasma and liver Hcy (R=0.60, p=0.09) correlated poorly and not significantly, whereas plasma and liver 5MTHF did not correlate at all (R=0.18, p=0.63).

Correlations of Hcy with other 1C metabolites in liver
After the control diet, liver Hcy correlated significantly with liver SAM (R=0.72, p=0.03). Liver Hcy did not correlate with liver SAH (R=0.08, p=0.83) and liver 5MTHF (R=-0.07, p=0.87).

Effects of diet on plasma and liver Hcy concentrations
The effect of the 2 intervention diets on plasma and liver Hcy concentrations is illustrated in Table 1 and Figure 2. The vitamin deficient diet increased plasma Hcy by almost 300% (p=0.00; $\eta^2=0.68$), but lowered liver Hcy by 25% (p=0.00; $\eta^2=0.46$). The high-methionine diet also increased plasma Hcy (+50%; p=0.00; $\eta^2=0.76$), but liver Hcy concentrations remained unchanged (p=0.18; $\eta^2=0.12$).

Figure 3. Effect of diet on liver CBS and MTHFR activity.

*Difference is significant at the 0.05 level (2-tailed). HM (high methionine diet), VD (vitamin deficient diet).
Table 1. 1C metabolite concentrations and enzyme activities for the different diet groups.

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>HM diet</th>
<th>VD diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>(Range)</td>
<td>Median</td>
</tr>
<tr>
<td>Hcy in plasma (µmol/L)</td>
<td>7.1</td>
<td>(5.7-8.5)</td>
<td>11.3**</td>
</tr>
<tr>
<td>Cystathionine in plasma (µmol/L)</td>
<td>0.25</td>
<td>(0.14-0.36)</td>
<td>0.32*</td>
</tr>
<tr>
<td>5MTHF in plasma (nmol/L)</td>
<td>35.0</td>
<td>(25.5-55.4)</td>
<td>35.7</td>
</tr>
<tr>
<td>Hcy in liver (nmol/mg protein)</td>
<td>1.32</td>
<td>(1.02-1.86)</td>
<td>1.27</td>
</tr>
<tr>
<td>Cystathionine in liver (nmol/mg)</td>
<td>0.13</td>
<td>(0.06-0.21)</td>
<td>0.31**</td>
</tr>
<tr>
<td>Met in liver (nmol/mg protein)</td>
<td>13.0</td>
<td>(10.1-21.2)</td>
<td>9.6**</td>
</tr>
<tr>
<td>SAM in liver (pmol/mg protein)</td>
<td>970</td>
<td>(735-1173)</td>
<td>1156</td>
</tr>
<tr>
<td>SAH in liver (pmol/mg protein)</td>
<td>255</td>
<td>(232-313)</td>
<td>252</td>
</tr>
<tr>
<td>SAM/SAH in liver</td>
<td>3.5</td>
<td>(3.0-5.0)</td>
<td>4.3</td>
</tr>
<tr>
<td>5MTHF in liver (pmol/mg protein)</td>
<td>98.9</td>
<td>(52.2-166.3)</td>
<td>102.1</td>
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<tr>
<td>Non-MTHF in liver (pmol/mg protein)</td>
<td>31.9</td>
<td>(23.1-72.0)</td>
<td>21.4</td>
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<td>MTHFR in liver (nmol/h/mg protein)</td>
<td>29.3</td>
<td>(12.3-40.0)</td>
<td>42.3*</td>
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<tr>
<td>CBS in liver (nmol/h/mg protein)</td>
<td>58</td>
<td>(18.22-81.6)</td>
<td>71.5*</td>
</tr>
</tbody>
</table>

VD (vitamin B deficient diet), HM (high methionine diet), non-MTHF (sum of THF, 5-formylTHF, 10-formylTHF, 5,10-methyleneTHF, and 5,10-methenylTHF)(Smith et al., 2006) *Difference is significant at the 0.05 level (2-tailed), **Difference is significant at the 0.01 level (2-tailed).
Effect of diet on other 1C metabolite concentrations in plasma and liver

The concentrations of all 1C metabolites in liver of the 3 different diet groups are shown in Table 1. The high-methionine diet (HM vs control) resulted in a significant increase in Cysta and decrease in Met concentrations in liver. The vitamin deficient diet (VD vs control) resulted in a significant lowering of Met, and non-methylTHF, and an increase of Cysta concentrations in liver (Table 1/Figure 2).

Effect of diet on 1C enzyme activities in liver

The CBS and MTHFR activities in liver of the 3 different diet groups are shown in Table 1 and Figure 3. The vitamin deficient diet (VD vs control) did not result in a significant change of CBS or MTHFR activity in liver. The high-methionine diet (HM vs control) resulted in a significant increase in both CBS (+20%; p=0.04) and MTHFR (+40%; p=0.03) activity in liver.

Discussion

Elevated plasma Hcy concentrations as a risk factor for numerous disorders have been extensively researched. However, exactly which organs/cells contribute to the plasma equilibrium, and whether plasma Hcy is at all an adequate reflection of intracellular Hcy, remains to be elucidated. This study focused on determining the correlation between plasma and liver Hcy (the most likely contributor to plasma Hcy), and addressed the effects of different Hcy-increasing diets on liver 1C metabolites and the 1C enzymes CBS and MTHFR in rats.

One important finding was the poor correlation between plasma Hcy and liver Hcy in the control group, which suggests, but not proves, that plasma Hcy does not originate from liver alone. Other organs probably play a role in plasma Hcy homeostasis, as was suggested previously.6 Probably, plasma Hcy is a multi-factorial equilibrium of uptake and export into and out of different cells/organs.

If plasma Hcy concentrations do not reflect intracellular Hcy adequately, measurement of plasma Hcy as a measure for the efficacy of folate, or other hcy-lowering therapies provides an incomplete picture of the impact of such treatments. This is supported by findings of hyperhomocysteinemia inducing diets (i.e. methionine excess or B-vitamin deficient) in this study. The anticipated elevation in plasma Hcy was indeed observed, but liver Hcy fell in
the vitamin deficient group, and remained unchanged after high-methionine. Whether a lowered 1C metabolic flux rate is responsible for these findings remains to be investigated. In any case, the discrepancy between plasma and intracellular Hcy might help explain why B-vitamin-based treatment thus far have failed to show benefit. Another indication that plasma concentrations are an inappropriate reflection of intracellular 1C metabolite dynamics is found in the correlation between the various 1C metabolites. Correlations between liver Hcy and other 1C metabolites are different than those previously published in plasma. In plasma, Hcy correlates with SAH and 5MTHF,\textsuperscript{19-21} In liver, we identified the plausible correlation of Hcy with SAM (high SAM concentrations lower MTHFR activity, and stimulate CBS activity, thus directly influencing Hcy concentrations\textsuperscript{22}). This correlation was found in human PBMCs before.\textsuperscript{15} This difference in 1C metabolite correlations intra- and extracellular might be explained by the fact that SAM is primarily an intracellular compound, which does not pass the cell membrane easily. In addition, 1C enzyme expression in liver is different than in most cells (i.e. CBS, betaine-homocysteine S-methyltransferase, glycine N-methyltransferase, different methionine adenosine transferase (MAT) isoforms), which may cause the equilibrium between SAH and Hcy to be less dominant. It should be kept in mind that the control rats in this study were fed folic acid (a synthetic analogue of folates, and a normal constituent of regular chow for animals in experimental conditions). This vitamin analogue may interfere with normal 1C metabolism in humans \textsuperscript{15}, which might cause concern for the interpretation of our results in rats. However, the appearance of folic acid in the circulation may be more persistent in humans, since dihydrofolate reductase activity is much lower in humans compared to rats (about 25 times \textsuperscript{23}). In the B-vitamin deficient diet, the anticipated decrease in plasma 5MTHF and a decrease in liver 5MTHF and non-methylTHF concentrations was observed, although this was not significant for liver 5MTHF. This may be due to a lack of power. Non-methylTHF concentrations on the other hand decreased dramatically. One possible interpretation is that, under low folate conditions, there is a preference to preserve methylation at the expense of 5MTHF concentrations. Liver Met concentrations were also significantly decreased while liver Cysta was increased. Maybe the lowered flux of the 1C cycle due to loss of co-factors pushes the Hcy towards the transsulfuration pathway or out of the cell. This lowered flux may also be responsible for the decrease in liver SAM and increase in liver SAH concentrations in the vitamin B deficient diet, which was also observed in previously published papers.\textsuperscript{24,25}
In the methionine enriched diet, one might expect that liver Met concentrations would rise when the Met content of the diet increases. The opposite was observed in this study. This might be explained by the interactions of the different methionine adenosyltransferase MAT isoforms in liver (unlike other tissues). MAT I is inhibited by elevated SAM concentrations, but on the other hand MAT III is activated by elevated SAM concentrations which may be a means of clearance of excess methionine. A high methionine diet has been shown to increase the activity of MAT I 4-5 fold while MAT II and III activity was increased only 1.5 fold. In addition, liver Hcy and SAM concentrations remained unchanged while liver Cysta concentrations were elevated about 2 fold. The elevated activity of both CBS and MTHFR might indicate an adaptation of the 1C cycle flux due to elevated dietary Met.

This study shows that plasma 1C metabolite concentrations are not simply a reflection of liver concentrations. Apparently, Hcy homeostasis in plasma is a complex equilibrium of uptake and export of different cells and organs. Two dietary interventions elevated plasma Hcy, but had different and sometimes unexpected effects on intracellular 1C metabolites, at least in liver. This indicates that the workings of 1C metabolism are more complex than meets the eye when just looking at plasma Hcy. We need to take a closer look at the results of B-vitamins at the cellular concentration to discover what exactly we have been studying over the last years, when plasma 1C metabolites were our focus.

References


