Mild hyperhomocysteinemia is an independent risk factor for cardiovascular (1, 2) and atherosclerotic disease (3). Total plasma homocysteine (Hcy) values of −10 μM for men and −8 μM for women are in the normal range. However, even a small increase (−5 μM) in total plasma Hcy is associated with a 60% increased risk of coronary artery disease for men and 80% for women (3). Moreover, a number of studies have demonstrated that smoking, excessive drinking of alcohol, obesity, type II diabetes, and an unhealthy diet contribute to mild hyperhomocysteinemia (1, 2). In addition, elevated plasma Hcy has recently been linked to Alzheimer’s disease and cognitive impairment in the elderly (4, 5).

Hcy is a non-protein amino acid derived from the catabolism of S-adenosylhomocysteine (AdoHcy), an immediate product of trans-methylation reactions that utilize S-adenosylmethionine (AdoMet) (6). Hcy has three possible fates: 1) methylation to methionine with N-5-methyltetrahydrofolate or betaine as the methyl donor, 2) conversion to cysteine via the trans-sulfuration pathway, and 3) release into extracellular fluids (e.g. plasma and urine). AdoMet-dependent methyltransferases catalyze many critical reactions including methylation of RNA, DNA, proteins, and small molecules such as guanidinoacetate and glycine (6). The potential of methyltransferases to regulate plasma Hcy is not well defined.

Phosphatidylethanolamine N-methyltransferase (PEMT) is a liver-specific enzyme that generates AdoHcy during the conversion of one membrane lipid, phosphatidylethanolamine, into another membrane lipid, phosphatidylcholine (PC) (7). PEMT accounts for the formation of −30% PC made in liver (8, 9). With this large capacity for PC synthesis and the generation of three AdoHcy molecules for each PC molecule synthesized, we hypothesized that the PEMT reaction might contribute significantly to Hcy in plasma. We have utilized the Pemt−/− mouse, hepatocytes derived from these mice, and overexpression of PEMT in McArdle RH7777 (rat hepatoma) cells to test this hypothesis. The results show that PEMT expression enhances plasma Hcy levels and the secretion of Hcy from hepatocytes.

**EXPERIMENTAL PROCEDURES**

Materials—For the high fat/high cholesterol diet, a semi-purified diet lacking a fat source was purchased from Teklad (catalog number 84712, Madison, WI) and supplemented with 19% (w/w) olive oil, 1% (w/w) linseed oil (a source of essential fatty acids), and 1% (w/w) cholesterol. Rodent chow was from LabDiet (PICO Laboratory Rodent Diet 20). Hanks’ balanced salt solution, Dulbecco’s Modified Eagle’s medium (DMEM), and fetal bovine serum were from Invitrogen. All other chemicals were from Sigma unless noted otherwise.

In Vivo Experiments—The Pemt−/− mouse colony had a mixed genetic background of 129S/J and C57BL/6 mice and was maintained by homozygous breeding in a reversed 12-h light/dark cycle. At the age of 12–14 weeks, Pemt−/− and Pemt+/− mice were fed ad libitum either chow or the high fat/high cholesterol diet for 3 weeks (10).

Primary Hepatocyte Cultures—Male Pemt−/− and Pemt+/− mice (12–20 weeks old) that were fed chow were used. Primary hepatocytes were isolated by collagenase perfusion (11) and plated on 60-mm collagen-coated dishes at a density of 2.0 × 10^6 cells/dish in DMEM containing 17% fetal bovine serum and 0.1 mg/ml insulin. After 7.5 h, the primary cultures were rinsed twice in serum-free DMEM over a 1-h period and then incubated in serum-free DMEM for 12 h at 37 °C. Medium and cells were collected and frozen at −70 °C until analysis. The cells were re-destructed and sonicated in homogenization buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonylfluoride). Protein analysis was performed with the Coomassie Blue Plus protocol from Bio-Rad.

Culture and Transfection of McArdle RH7777 Rat Hepatoma Cells—McArdle RH7777 rat hepatoma cells stably expressing human PEMT cDNA or vector alone (pCI, Promega) were maintained in DMEM con-
taining 20% fetal bovine serum, 200 µg/ml gentamycin, and 100 µM ethanolamine. The ethanolamine was added to ensure that there was sufficient substrate for phosphatidylethanolamine biosynthesis so that this lipid would not be limiting for phospholipid methylation. Following a 24-h incubation period, the medium was removed, centrifuged to remove cell debris, and frozen until analysis.

Measurement of Amino Acid Concentrations and Enzyme Activities—Livers were removed from mice and flash-frozen. The protein content of the liver samples was determined by the Biuret method (13). Thawed liver samples were homogenized in 50 mM phosphate-buffered saline (pH 6.9). The homogenate was centrifuged at 18,000 × g for 30 min at 4 °C, and the following enzyme activities were measured in the supernatant: cystathionine β-synthase (14, 15), AdoMet synthase (15), methionine synthase (16), 5,10-methylenetetrahydrofolate reductase (17), and betaine:homocysteine methyltransferase (18). The final product of betaine:homocysteine methyltransferase, methionine, was measured by reverse-phase high performance liquid chromatography.

Total Hcy content in plasma and media was measured by reverse-phase high performance liquid chromatography and fluorescence detection of ammonium 7-fluoro-2-oxa-1,3-diazole-4-sulfonate thiol adducts (12).

The amounts of methionine, total cysteine, serine, and glycine in media were measured in samples that had been deproteinized by treatment with 10% sulfosalicylic acid. The protein was removed by centrifugation, and the pH of the supernatant was adjusted to 2.2. The amino acids were analyzed on a Beckman 121 MB amino acid analyzer using Bensen n-X 0.25 Cation Xchange Resin according to Beckman 121MB-TB-O17 application notes and quantitated using a Hewlett Packard Computing Integrator Model 3395A following post-column derivatization with ninhydrin. PEMT activity was measured as described previously (19).

RESULTS

Pemt<sup>−/−</sup> Mice Have Decreased Plasma Levels of Homocysteine—Female and male Pemt<sup>+/+</sup> and Pemt<sup>−/−</sup> mice were fed chow or a high fat/high cholesterol (HF/HC) diet for 3 weeks. Results are the means ± S.D. from at least four mice, *p < 0.001 for Pemt<sup>−/−</sup> compared with Pemt<sup>+/+</sup> mice based on a one-way ANOVA followed by a Newman-Keuls post-test.

![Graph showing plasma Hcy levels in Pemt<sup>−/−</sup> mice compared to Pemt<sup>+/+</sup> mice.](image)

**Fig. 1. Plasma Hcy levels are lower in Pemt<sup>−/−</sup> mice than in Pemt<sup>+/+</sup> mice.** Plasma Hcy levels were measured in Pemt<sup>+/+</sup> and Pemt<sup>−/−</sup> mice that were fed chow or a high fat/high cholesterol (HF/HC) diet for 3 weeks. Results are the means ± S.D. from at least four mice. *p < 0.001 for Pemt<sup>−/−</sup> compared with Pemt<sup>+/+</sup> mice based on a one-way ANOVA followed by a Newman-Keuls post-test.

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RESULTS

Pemt<sup>−/−</sup> Mice Have Decreased Plasma Levels of Homocysteine—Female and male Pemt<sup>+/+</sup> and Pemt<sup>−/−</sup> mice were fed chow or a high fat/high cholesterol diet for 3 weeks. Fig. 1 shows that the plasma content of Hcy in Pemt<sup>−/−</sup> mice was ~50% less than in Pemt<sup>+/+</sup> mice. Neither gender nor diet influenced the levels of plasma Hcy.

To determine whether alterations of activities of other key hepatic enzymes of homocysteine production and removal might have caused the decreased plasma Hcy levels in Pemt<sup>−/−</sup> mice, we assayed five enzymes listed in Table I. The activities were not statistically different between Pemt<sup>−/−</sup> and Pemt<sup>+/+</sup> mice. Hence, the 50% lower level of plasma Hcy in the Pemt<sup>−/−</sup> mice is probably because of the absence of PEMT activity in the liver.

Decreased Secretion of Homocysteine from Hepatocytes Derived from Pemt<sup>−/−</sup> Mice—We measured the secretion of Hcy from primary hepatocytes isolated from Pemt<sup>+/+</sup> and Pemt<sup>−/−</sup> mice. Fig. 2 shows that hepatocytes from Pemt<sup>−/−</sup> mice secrete ~50% less Hcy than hepatocytes from Pemt<sup>+/+</sup> mice. It was conceivable that the depletion of intracellular AdoMet decreased Hcy secretion from Pemt<sup>−/−</sup> hepatocytes. To test this possibility, we performed a parallel experiment in which hepatocytes were incubated with guanidinoacetate. Methylation of guanidinoacetate to form creatine is an important contributor to the exported Hcy pool (20). Therefore, if AdoMet was sufficient in the hepatocytes from Pemt<sup>−/−</sup> mice, we should observe enhanced Hcy secretion. Fig. 2 indicates that guanidinoacetate-stimulated Hcy secretion from both Pemt<sup>+/+</sup> and Pemt<sup>−/−</sup> hepatocytes to a similar degree. Thus, apparently a lack of AdoMet seems unlikely. These results concur with previous measurements of the levels of AdoMet (~130 pmol/mg liver) and AdoHcy (~40 pmol/mg liver) that were similar in the livers of Pemt<sup>+/+</sup> and Pemt<sup>−/−</sup> mice (21). We also established at the end of the incubation period that methionine, cysteine, serine, and glycine had not been depleted from the medium and therefore were not limiting Hcy secretion. Thus, AdoMet was not limiting for Hcy formation, and the decreased secretion of Hcy from Pemt<sup>−/−</sup> hepatocytes was attributed to the lack of PEMT.

Plasma homocysteine is also increased in rats that were provided with dietary guanidinoacetate as is the production of homocysteine by hepatocytes incubated with guanidinoacetate (20). These results demonstrate that altered flux through another major methyltransferase can also affect homocysteine metabolism.

**Expression of PEMT Stimulates Secretion of Homocysteine from Hepatoma Cells—McArdle RH-7777 hepatoma cells have negligible PEMT activity (19). We reasoned that if Hcy secretion were dependent upon PEMT activity, stable expression of PEMT in these cells would stimulate Hcy secretion. Fig. 3 confirms the low PEMT activity in parental McArdle cells and indicates that the secretion of Hcy from cells that expressed human PEMT was enhanced. We also demonstrated that methionine, cysteine, serine, and glycine had not been depleted from the medium at the end of the incubation period.**

**DISCUSSION**

If ~50% plasma Hcy was derived from the PEMT reaction as suggested by our results, PEMT must generate significant amounts of AdoHcy in the liver. A 20-g mouse secretes ~30 pmol (23 mg) PC into bile each day (22), and the PEMT pathway is an important source for PC in the bile (23). Approximately, one-third of the PC in murine liver is derived from the PEMT pathway (8, 9). Therefore, ~10 µmol biliary PC should be produced via PEMT in 24 h. Each phosphatidylethanolamine molecule methylated to PC produces three molecules of AdoHcy. Hence, to satisfy the export of PC into bile, the murine liver produces ~30 µmol AdoHcy in 24 h from the PEMT reaction. This estimate does not take into account the presumed sizeable requirement of PC biosynthesis for hepatocyte membranes or for export with lipoproteins.

From Fig. 2, we estimated the amount of Hcy secreted from 1 g of liver over a 24-h period. Murine hepatocytes secreted 15 nmol Hcy/mg protein over a 12-h period. Assuming that a 1-g liver yields 175 mg of protein (24), a 1-g liver would produce 5.25 µmol Hcy/day. Although these calculations are approximations, it is clear that hepatic PEMT generates significantly more AdoHcy (>30 µmol) than Hcy (~5 µmol) secreted from the liver each day.

In addition to defining a role for PEMT in the regulation of plasma Hcy, the work also argues for a primary role of the liver in producing Hcy. All of the nucleated cells have the capacity to generate AdoHcy and probably Hcy; hence, it is difficult to determine the quantitative source of plasma Hcy. Because PEMT is quantitatively abundant only in the liver (7), our data...
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Specific activities of enzymes involved in methionine and homocysteine metabolism

The results are mean values ± S.D. for 3–5 animals that were fed a chow diet. Similar values were obtained from mice that were fed a high fat/high cholesterol diet. Differences between Pemt+/+ and Pemt−/− mice were analyzed using ANOVA followed by Newman-Keuls test, but no significant differences were found.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Pemt+/+</th>
<th>Pemt−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystathionine β-synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>11.1 ± 2.4</td>
<td>11.7 ± 2.4</td>
</tr>
<tr>
<td>Females</td>
<td>13.5 ± 2.7</td>
<td>13.4 ± 1.2</td>
</tr>
<tr>
<td>AdoMet synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Females</td>
<td>0.92 ± 0.1</td>
<td>0.78 ± 0.2</td>
</tr>
<tr>
<td>Methionine synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>0.063 ± 0.004</td>
<td>0.054 ± 0.015</td>
</tr>
<tr>
<td>Females</td>
<td>0.026 ± 0.005</td>
<td>0.029 ± 0.002</td>
</tr>
<tr>
<td>Methylenetetrahydrofolate reductase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>0.027 ± 0.006</td>
<td>0.031 ± 0.004</td>
</tr>
<tr>
<td>Females</td>
<td>0.037 ± 0.008</td>
<td>0.033 ± 0.008</td>
</tr>
<tr>
<td>Betaine:Hcy methyltransferase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>3.7 ± 0.4</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>Females</td>
<td>3.8 ± 0.3</td>
<td>3.8 ± 0.6</td>
</tr>
</tbody>
</table>

![Fig. 2. Less Hcy is secreted from Pemt−/− than from Pemt+/+ hepatocytes.](image)

Hepatocytes were isolated from Pemt+/+ and Pemt−/− mice. After a 12-h incubation with guanidinoacetate (GA), the media were collected and frozen, and the amount of Hcy was determined. The differences among all of the four variables were significant (p < 0.001) by ANOVA followed by a Newman-Keuls test-post-test.

The results demonstrate that alterations in plasma Hcy in mice are largely mediated by the liver. Moreover, the hepatic methylation of guanidinoacetate to creatine to replace urinary creatinine loss (estimated at 3–15 μmol/day in mice (25, 26)) is also considered a source of plasma Hcy.

Much of what is known concerning AdoMet consumption in trans-methylation reactions is derived from the elegant studies of Mudd et al. (27, 28) on the balance of methyl groups in humans. These experiments estimated that creatine synthesis in the liver consumes ~75% of available AdoMet. Of the remaining 25% AdoMet, ~15% was estimated to be used for PC synthesis from phosphatidylethanolamine and the remaining ~10% was estimated to be used for other trans-methylation and polyamine synthesis. In those studies, the measurement of AdoMet consumption via PEMT involved the oxidation of choline to sarcosine and the recovery of this compound in urine.

When sarcosine was administered to a patient deficient in sarcosine dehydrogenase, only 60–80% was recovered in urine. Therefore, the recovery of sarcosine in the urine in these calculations underestimated its formation and, thus, the contribution of the PEMT reaction. Moreover, because many destinations require PEMT-derived PC (e.g. cellular membranes, bile secretion, lipoprotein secretion, sphingomyelin synthesis), the measurement of the oxidation of choline to sarcosine significantly underestimates the consumption of AdoMet in the PEMT reaction. Our results suggest that PC synthesis from phosphatidylethanolamine consumes substantially more AdoMet than was previously thought. Mudd et al. (27, 28) note that their estimates must be revised as more data become available. It appears that such revision is now appropriate.

The results demonstrate that the PEMT reaction in liver is a major source of plasma Hcy, an independent risk factor for cardiovascular disease. Several studies (29, 30) have demonstrated that hyperhomocysteinemia is detrimental to normal vascular endothelial function. Furthermore, elevated Hcy levels stimulate vascular smooth muscle cell growth and migration as well as the recruitment of monocytes to atherosclerotic lesions (31–34). Although Hcy enhances the development of atherosclerotic lesions, it has not been demonstrated that Hcy is a cause of atherosclerosis. Studies in patients with mild hyperhomocysteinemia but without any other cardiovascular disease risk factors were shown to be at no greater risk for the
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disease compared with healthy individuals (35). Also, neither cystathionine β-synthase nor methylene tetrahydrofolate reductase-deficient mice developed atherosclerosis until old age, even though the mice had severe homocysteinuria (36).

Regardless of whether or not mild hyperhomocysteinemia causes or merely correlates with the development of cardiovascular disease, it is a strong predictor of mortality in individuals (37). It is now shown that the PEMT reaction in the liver is a major source of plasma Hcy. A previous study (38) with hepato-
cytotoxicants derived from Pemt-/- mice demonstrates that PEMT is also important in regulation of the secretion of apolipoprotein B-containing lipoproteins, high levels of which leads to cardiovascular disease. Therefore, continued research on the role of PEMT in the generation of plasma Hcy will lead to a better understanding of the possible relationship between mild hyper-

homocysteinemia and other cardiovascular risk factors.

Acknowledgments—We thank Sandra Ungurian, Susanne Lingrell, and Laura Hargreaves for excellent technical assistance and Dr. René Jacobs, Dr. David Wood, Dr. Martin Raabe, and Dr. Jean Vance for helpful discussions.

REFERENCES
