Physiological Regulation of Phospholipid Methylation Alters Plasma Homocysteine in Mice*

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Biological methylation reactions and homocysteine (Hcy) metabolism are intimately linked. In previous work, we have shown that phosphatidylethanolamine N-methyltransferase, an enzyme that methylates phosphatidylethanolamine to form phosphatidylcholine, plays a significant role in the regulation of plasma Hcy levels through an effect on methylation demand (Noga, A. A., Stead, L. M., Zhao, Y., Brosnan, M. E., Brosnan, J. T., and Vance, D. E. (2003) J. Biol. Chem. 278, 5852–5855). We have further investigated methylation demand and Hcy metabolism in liver-specific CTP:phosphocholine cytidylyltransferase-α (CTa) knockout mice, since flux through the phosphatidyl ethanolamine N-methyltransferase pathway is increased 2-fold to meet hepatic demand for phosphatidylcholine. Our data show that plasma Hcy is elevated by 20–40% in mice lacking hepatic CTa. CTa-deficient hepatocytes secrete 40% more Hcy into the medium than do control hepatocytes. Liver activity of betaine-homocysteine methyltransferase and methionine adenosyltransferase are elevated in the knockout mice as a mechanism for maintaining normal hepatic S-adenosylmethionine and S-adenosylhomocysteine levels. These data suggest that phospholipid methylation in the liver is a major consumer of AdoMet and a significant source of plasma Hcy.

Elevations in plasma homocysteine (Hcy),1 a nonprotein sulfur-containing amino acid, is an independent risk factor for cardiovascular (1, 2) and atherosclerotic diseases (3). In humans, total plasma Hcy concentration normally ranges from 8 to 12 μM. However, a small elevation (~5 μM) in plasma Hcy increases the risk of coronary artery disease by as much as 60% in men and 80% in women (3). Hyperhomocysteinemia has also been correlated with smoking, obesity, diabetes, hypertension, and impaired B vitamin status (1, 2). Furthermore, altered Hcy metabolism has been observed in Alzheimer’s disease (4) and in the elderly with cognitive impairment (5).

Homocysteine is formed during the metabolism of methionine. Methionine is activated by methionine adenosyltransferase to form S-adenosylmethionine (AdoMet), an important biological methyl donor (6). Numerous methyltransferases catalyze the transfer of the methyl group from AdoMet to a methyl acceptor, producing a methylated product and N5-methyltetrahydrofolate, catalyzed by methionine synthase (MAT), both of which are methyl donors. The catabolism of Hcy is accomplished by the transsulfuration pathway, which consists of two enzymes, cystathionine β-synthase and cystathionine γ-lyase. Finally, Hcy can be secreted from cells into the circulation.

It is clear that biological methylation and Hcy metabolism are closely related. However, the potential of specific methyltransferases to regulate plasma Hcy levels has been understudied. We have previously utilized the phosphatidylethanolamine N-methyltransferase (PEMT) knockout mouse to investigate the importance of phospholipid methylation in Hcy metabolism (7). PEMT is a liver-specific enzyme that methylates one membrane lipid, phosphatidylethanolamine (PE), into another membrane lipid, phosphatidylcholine (PC) (8). Three AdoMet molecules are consumed in the course of this reaction. Since PEMT accounts for 30% of hepatic PC synthesis (9–11), we hypothesized that deletion of the gene would significantly alter Hcy metabolism. Indeed, plasma Hcy was reduced by 50% in Pemt−/− mice compared with wild type controls (7). Moreover, hepatocytes isolated from Pemt−/− mice secrete less Hcy into the medium, providing clear evidence that Hcy production is reduced in the knockout mice. These studies illustrate the potential for PEMT to regulate plasma Hcy.

Since there is no PEMT activity in the Pemt−/− mouse (12), it is impossible to use this model to investigate how increased physiological demand for phospholipid methylation affects Hcy metabolism. As noted above, PEMT activity accounts for 30% of hepatic PC biosynthesis. The remaining 70% of hepatic PC is produced from choline by the enzymes of the Kennedy (CDP-choline) pathway (9–11), and flux through this pathway is regulated by the activity of CTP:phosphocholine cytidylyltransferase.
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ferase (CT) (13–15). Hepatic cells express two CT isoforms (16), CTα (major expression), and CTβ (minor expression). We have generated a mouse model in which CTα is selectively deleted in the liver (17). In liver-specific CTα knockout mice, hepatic CT activity is 15% of normal, whereas PEMSCT activity is elevated 2-fold compared with controls. We hypothesized that induction of PEMT, while necessary to maintain close to normal hepatic PC levels, would increase hepatic Hcy production and result in elevated plasma Hcy. The results describe an important role for PEMT-derived PC biosynthesis in mice deficient in hepatic CTα and furthermore illustrate how physiological changes in PC metabolism influence Hcy metabolism.

MATERIALS AND METHODS

In Vivo Experiments—All procedures were approved by the University of Alberta’s Institutional Animal Care Committee and were in accordance with guidelines of the Canadian Council on Animal Care. The liver-specific CTα knockout mouse was generated using the Cre/Lox technology as previously described (17). All mice (12–20 weeks old) were fed ad libitum a chow diet (LabDiet, PICO laboratory Rodent Diet 20) and were exposed to a 12 h light/dark cycle starting at 8:00 a.m.

Cultures—Primary hepatocytes were isolated by collagenase perfusion (18) and plated on 60-mm collagen-coated dishes at a density of 1.0 × 10⁶ cells/dish in Dulbecco’s modified Eagle’s medium containing 30% fetal bovine serum. 2–4 h after plating, the cultures were rinsed twice in serum-free Dulbecco’s modified Eagle’s medium over a 1-h period and then incubated in serum-free Dulbecco’s modified Eagle’s medium containing 10 μM choline and 50 μM methionine for 10 h. Medium was collected, and cells were sonicated in phosphate-buffered saline (pH 7.4). The resultant supernatant was centrifuged at 100,000 g for 10 min to pellet unbroken cells and nuclei. The supernatant was adjusted to 2.2. The amino acids were analyzed on a Shimadzu HPLC system (Montreal Biotech). For comparison, a 10% SDS-polyacrylamide gel. Proteins were transferred to nylon membranes and probed with anti-BHMT1 (1:5000), anti-MAT1 (1:1200), or anti-protein-disulfide isomerase (1:5000) antibody.

Immunoblotting of Hepatic BHMT1 and MAT1—15 μg of liver homogenate was boiled in SDS buffer, and proteins were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to nylon membranes and probed with anti-BHMT1 (1:5000), anti-MAT1 (1:1200), or anti-protein-disulfide isomerase (1:5000) antibody. Immunoreactive bands were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences). Quantification of the immunoreactive bands was performed using Image Gauge version 3.0 software by Fuji.

RESULTS

PC Synthesis from the CDP-choline Pathway Is Impaired in CTα-deficient Hepatocytes—The Kennedy (CDP-choline) pathway has been shown to produce ~70% of PC in a normal liver (9–11). Disruption of the hepatic CTα gene resulted in an 85% reduction in total CT activity (17). The active form of CT in cells is considered to be associated with membranes, whereas the soluble form of CT is thought to be an inactive reservoir (30, 31). Movement of CT to and from the membrane is linked to cell requirements for PC and is tightly regulated (32–36). Interestingly, all hepatic CT activity was found in the membrane frac-
tion in the knockout mice; however, the active form of CT was still only 15% of control levels (Fig. 1A). We sought to determine to what extent the decrease in CT activity in the knockout mice was reflected in flux through the CDP-choline pathway. To that end, we incubated primary hepatocytes with [3H]choline for 2 h and measured radiolabeled phosphocholine (a substrate for the CT reaction) and PC. We observed that the amount of [3H]phosphocholine was 2-fold higher in the knockout hepatocytes than in control hepatocytes (Fig. 1B). Furthermore, the amount of newly formed PC was 65% less in hepatocytes deficient in CT (Fig. 1B). These data provide clear evidence for impaired flux through the CDP-choline pathway in livers deficient in CT.

Increased PE Methylation and Homocysteine Secretion from CTα-deficient Hepatocytes—Despite the significant reduction in PC biosynthesis by the CDP-choline pathway, hepatic PC levels were only slightly lower (10–20%) in CTα-deficient mice than in their wild-type counterparts (17), suggesting that PE methylation may be compensating for the decreased CT activity. Indeed, in our previous work, we reported a 2-fold higher PEMT activity in the knockout mice that might have resulted from increased PE methylation driven by the absence of CTα. Therefore, we assayed the major enzymes involved in the production (transmethylation) and removal (transsulfuration and remethylation) of Hcy in the liver. The activity of BHMT, which synthesizes AdoMet, was 2-fold higher in male knockout mice compared with gender-matched controls (Table I). Similarly, the activity of MAT, which synthesizes AdoMet, was 2-fold higher in both male and female knockout mice. CTα deficiency did not alter the activity of methionine synthase, methylenetetrahydrofolate reductase, or cystathionine β-synthase in the livers (Table I).

Our model provides the opportunity to examine whether or not the physiological increase in flux through the PEMT pathway, driven by the absence of CTα, results in elevated Hcy secretion from hepatocytes. We therefore measured the secretion of Hcy from primary hepatocytes isolated from control and knockout mice. Following a 2–10-h incubation period, hepatocytes from knockout mouse secreted 40% more Hcy into the media than did hepatocytes from control mice whether measured by radioactivity (Fig. 3A) or by mass (Fig. 3B). These data indicate that a physiological enhancement of methylation demand does indeed increase Hcy secretion from hepatocytes.

FIG. 1. Flux through the Kennedy (CDP-choline) pathway is impaired in CTα-deficient hepatocytes. A, liver homogenates, membranes, and cytosol from mice were assayed for CT activity. Values are means ± S.D. (n = 4–6 mice). B, primary hepatocytes were incubated with [3H]choline for 2 h. Cells were collected, and the radiolabel in choline, phosphocholine, and PC was determined. Values are means ± S.D. from three independent experiments. The asterisks signify differences versus control (p < 0.05).

FIG. 2. PE methylation is increased in CTα-deficient hepatocytes. Hepatocytes were isolated from control and hepatic CTα-deficient mice and incubated with [3H]ethanolamine (A) or [methyl-3H]methionine (B) for 0.5–2 h. Medium and cells were collected, and the radiolabel in PE and PC was determined. Data are means ± S.D. from 3–4 separate hepatocyte preparations. The asterisks signify differences versus control, p < 0.05.

Plasma Levels of Hcy Are Increased in Hepatic CTα-deficient Mice—Fig. 4 shows that mice deficient in hepatic CTα have higher levels of plasma Hcy than do control mice. We observed a 20% higher level of plasma Hcy in male knockout mice than in male control mice, whereas in female knockout mice, plasma Hcy was 40% higher than in their female littermates. The plasma concentration of methionine, cysteine, serine, glycine, and taurine were not significantly affected by genotype in either male or female mice (data not shown).

The increase in plasma Hcy, coupled with an increase in Hcy secretion from the knockout hepatocytes, suggests that CTα deficiency in the liver appreciably alters Hcy metabolism. Therefore, we assayed the major enzymes involved in the production (transmethylation) and removal (transsulfuration and remethylation) of Hcy in the liver. The activity of BHMT, which synthesizes AdoMet, was 2-fold higher in both male and female knockout mice. CTα deficiency did not alter the activity of methionine synthase, methylenetetrahydrofolate reductase, or cystathionine β-synthase in the livers (Table I). To address the possible mechanism(s) for the increased activity of MAT and BHMT, we measured the mRNA levels of their respective genes by real-time PCR (Fig. 5A). Bhm1, Mat1α, and Pemt mRNA levels were increased by 2.2–1.7, and 2.4-fold, respectively, as compared with controls. However, the
mRNA levels of *Bhmt*2 and *Mat2a* were unaltered. Protein levels of BHMT1 and MAT1 were also elevated in knockout mice. These data suggest that betaine (choline) oxidation is important in regulating Hcy production and, in a broader sense, the role of methylation demand. The level of plasma Hcy is 50% lower in *Pemt*−/− mice compared with wild-type mice, suggesting that flux through the PEMT pathway is an important determinant of plasma homocysteine levels (7). Our current report builds substantially on this initial observation. PC is vital for the structural integrity of mammalian membranes and, in the liver, is secreted into the bile as a constituent of lipoproteins. Under normal circumstances, the CDP-choline pathway produces 70% of hepatic PC (9–11). However, in liver-specific CTα knockout mice, PC synthesis via the hepatic CDP-choline pathway is reduced to 20% of control levels (Fig. 1B). In order to compensate for the lack of PC biosynthesis, PEMT mRNA (Fig. 5A), protein, and activity (17) are induced in the CTα-deficient livers, resulting in an increased flux through the PEMT pathway in hepatocytes. Importantly, these changes occur without manipulation of dietary substrates (methionine or choline), which allowed us to study directly the extent increased PE methylation modulates Hcy metabolism. We show, for the first time, that physiological stimulation of PEMT increases production of Hcy in the liver and, consequently, increases the level of plasma Hcy. These results are consistent with earlier experiments in cultured hepatocytes in which a direct relationship was shown between the level of PEMT expression and homocysteine export.

Our data in the PEMT and the liver-specific CTα knockout mice challenge the long held assertion that creatine synthesis is the major consumer of AdoMet molecules and, therefore, the main methylation reaction involved in the generation of Hcy (6). Other studies provide data to support our assertion. Reo *et al.* (10) have estimated a rate of synthesis of PEMT-derived PC in rats to be 0.15–0.21 μmol/kg/h liver. When this number is multiplied by 10 (10 g of liver in a 250-g rat) and by 24 (for a 24-h day), the rate of PC synthesis via the PEMT reaction is 36–50 μmol/250-g rat/day. Since three AdoMet molecules are consumed for each PC molecule synthesized, the amount of AdoMet utilized by PEMT would, therefore, be 108–150 μmol/250-g rat/day. Creatine biosynthesis begins with the synthesis of guanidinoacetate in the kidney. 1-Arginine:glycine amidino-
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TABLE I

Specific activities of enzymes involved in methionine and homocysteine metabolism

Livers from liver-specific CTk knockout and control mice were homogenized, and enzymatic activities were measured in the homogenates. The results are means ± S.D. for 5–9 animals.

| Enzyme                                | Control | Knockout |
|---------------------------------------|---------|----------|
| Cystathionine β-synthase               |         |          |
| Males                                 | 11.4 ± 2.9 | 11.5 ± 2.9 |
| Females                               | 8.9 ± 3.2  | 10.0 ± 3.9 |
| Methionine adenosyltransferase         |         |          |
| Males                                 | 0.44 ± 0.1  | 1.1 ± 0.3* |
| Females                               | 0.35 ± 0.12 | 0.76 ± 0.15* |
| Methionine synthase                    |         |          |
| Males                                 | 0.31 ± 0.04 | 0.29 ± 0.03 |
| Females                               | 0.25 ± 0.06 | 0.26 ± 0.05 |
| Methylene tetrahydrofolic reductase    |         |          |
| Males                                 | 0.061 ± 0.004 | 0.061 ± 0.005 |
| Females                               | 0.066 ± 0.005 | 0.064 ± 0.008 |
| Betaine:homocysteine methyltransferase |         |          |
| Males                                 | 0.59 ± 0.13  | 0.83 ± 0.16* |
| Females                               | 0.44 ± 0.10  | 0.75 ± 0.09* |

* Differences versus gender-matched controls, p < 0.05.

Fig. 5. Hepatic expression of BHMT1, Mat1a, and Pemt is increased in liver-specific CTk knockout mice. A, the Pfaffl method was utilized to determine relative mRNA levels using cyclophilin as the housekeeping gene. Means ± S.D. for three samples are shown. The asterisks signify differences versus control, p < 0.05. B, hepatic proteins were fractionated by SDS-polyacrylamide gel electrophoresis and immunoblotting was performed using antibodies raised against BHMT1, MAT1, and protein-disulfide isomerase (PDI). For each measurement, there were three samples from each test group. Intensities of band were quantitated using Image Gauge version 3.0 software by Fuji and the relative levels of BHMT1 and MAT1 were compared with PDI.

Methionine methyltransferase catalyzes the transfer of the amidino group of arginine to glycine, yielding ornithine and guanidinoacetate. Guanidinoacetate is carried in the blood to the liver, where it is methylated by AdoMet via guanidinoacetate N-methyltransferase to form creatine, which is then exported from the liver to extrahepatic tissues. Creatine and creatine phosphate are converted nonenzymatically to creatinine, the excreted form. In rodents, creatinine excretion has been measured at a rate of 58 μmol/250-g rat/day (45). If the rate of creatinine excretion equaled the rate of creatine synthesis (a reasonable assumption), PEMT would consume at least 2–3 times more AdoMet than would guanidinoacetate N-methyltransferase and thus be a quantitatively more important source of plasma Hcy. We have previously studied the contribution of creatine synthesis in regulating plasma Hcy in rats (46). We hypothesized that if creatine synthesis accounted for the majority of AdoMet consumed, the provision of creatine in the diet would decrease plasma Hcy. We observed a 90% reduction in arginine:glycine amidinotransferase activity (the enzyme that synthesizes guanidinoacetate) but only a ~20% reduction in plasma Hcy, less than would be anticipated if creatine synthesis accounted for 75% of AdoMet consumption (6). In contrast, deletion of PEMT in mice reduced the level of plasma Hcy by 50% (7), a much greater reduction than would have been expected if PE methylation consumed only 15% of AdoMet (6).
way can reduce hepatic Hcy secretion, thereby masking Hcy generation by PEMT. We have now observed that BHMT activity was increased by 80% in the C\textalpha\ knockout mice (Table I). Furthermore, we have provided evidence that choline (betaine) is an excellent source of methyl groups in the form of methionine. The hepatic PEMT reaction is a major producer of Hcy, an activity was increased by 80% in the C\textalpha\ knockout mice (Table I). Furthermore, we have provided evidence that choline (betaine) is an excellent source of methyl groups in the form of methionine.

Recent studies showed that deletion of PEMT reduces atherosclerotic plaque formation in the \textit{Ldr}^{-/-} (low density lipoprotein receptor) knockout mice by lowering the hepatic secretion rate of very low density lipoprotein (67) and thus the level of plasma cholesterol.\textsuperscript{3} Hepatic Hcy secretion is also reduced in \textit{Pemt}^{-/-} compared with \textit{Pemt}^{+/+} mice (7), and it is possible that the resulting decrease in plasma Hcy plays a role in the reduction of atherosclerotic lesions. Alternatively, the level of Hcy might represent a marker of atherosclerosis. In our future work, we shall determine if elimination of C\textalpha\ in the liver affects the development of atherosclerosis, since these mice have a reduction in plasma cholesterol (17) but an increase in plasma Hcy levels.

Conclusions—Induction of PEMT in the liver of hepatic C\textalpha\-deficient mice results in a 20–40% increase in plasma Hcy. We have previously shown that deletion of PEMT results in a 50% reduction in plasma Hcy (7). Taken together, it is now clear that regulation of hepatic PEMT is not only important in producing PC but is also a major source of plasma Hcy.

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