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QUARTERLY

Review

Methylation demand: a key determinant of homocysteine metabolism★✪

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Elevated plasma homocysteine is a risk factor for cardiovascular disease and Alzheimer's disease. To understand the factors that determine the plasma homocysteine level it is necessary to appreciate the processes that produce homocysteine and those that remove it. Homocysteine is produced as a result of methylation reactions. Of the many methyltransferases, two are, normally, of the greatest quantitative importance. These are guanidinoacetate methyltransferase (that produces creatine) and phosphatidylethanolamine *N*-methyltransferase (that produces phosphatidylcholine). In addition, methylation of DOPA in patients with Parkinson's disease leads to in-

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Abbreviations: AGAT, L-arginine:glycine amidinotransferase; BHMT, betaine:homocysteine methyltransferase; CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; COMT, catechol-*O*-methyltransferase; L-DOPA, L-3,4-dihydroxyphenylalanine; GAA, guanidinoacetate; GAMT, guanidinoacetate methyltransferase; MAT, methionine adenosyltransferase; MS, methionine synthase; MT, methyltransferase; MTHFR, methylene tetrahydrofolate reductase; PC, phosphatidylcholine; PEMT, phosphatidylethanolamine *N*-methyltransferase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; tHcy, total plasma homocysteine; THF, tetrahydrofolate.

creased homocysteine production. Homocysteine is removed either by its irreversible conversion to cysteine (transsulfuration) or by remethylation to methionine. There are two separate remethylation reactions, catalyzed by betaine:homocysteine methyltransferase and methionine synthase, respectively. The reactions that remove homocysteine are very sensitive to B vitamin status as both the transsulfuration enzymes contain pyridoxal phosphate, while methionine synthase contains cobalamin and receives its methyl group from the folic acid one-carbon pool. There are also important genetic influences on homocysteine metabolism.

Homocysteine, a non-protein amino acid, normally occurs in the plasma at a concentration of about 8–12 μM. The nature of plasma homocysteine is complex (Jacobsen, 2001). Only 1–2% is found as free (reduced) homocysteine. Approximately 20% occurs as free, oxidized forms, principally the mixed disulfide, cysteinylhomocysteine, but also as homocystine. The remainder, approximately 80%, is protein-bound; the great bulk of this is linked to cysteine-34 of albumin by a disulfide linkage (Jacobsen, 2001). These forms of plasma homocysteine are shown in Fig. 1. The sum of these forms of plasma homocysteine, total homocysteine, is designated "tHcy".

Homocysteine has been implicated as a risk factor for a number of important diseases. Increased tHcy (hyperhomocysteinemia) has been recognized as an independent risk factor for the development of vascular disease (Boushey *et al.*, 1995). Recent findings show a relationship between elevated tHcy and neurodegenerative diseases, in particular with Alzheimer's disease (Seshadri *et al.*, 2002; Clarke *et al.*, 1998). Relatively small changes in tHcy may dispose to pathological outcomes. Boushey *et al.* (1995) have calculated that as little as a 5 μM elevation can increase the risk of coronary artery disease by as much as 60% in men and 80% in women. For this reason, it is important to understand homocysteine metabolism and to identify the factors that determine plasma levels of this amino acid.

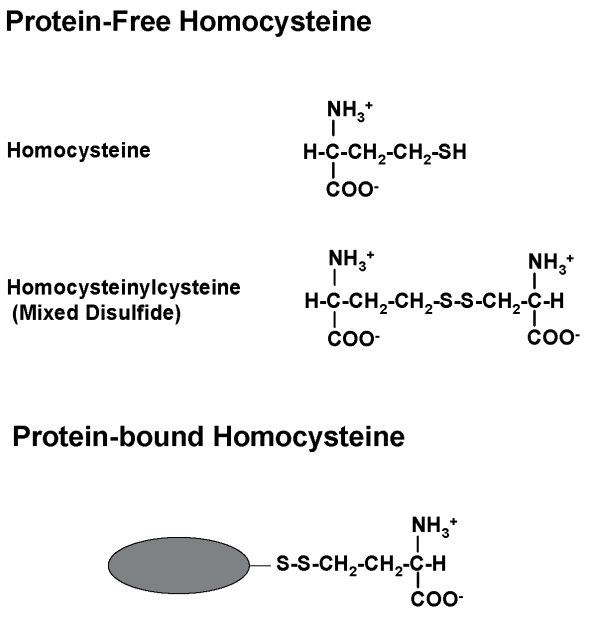

Figure 1. Species of homocysteine in plasma.

OUTLINE OF HOMOCYSTEINE METABOLISM

Homocysteine arises during methionine metabolism (Fig. 2). Methionine is activated to *S*-adenosylmethionine (SAM) by the enzyme methionine adenosyltransferase (EC 2.5.1.6). SAM is the major biological methyl donor; at least 39 methyltransferases have been identified (Clarke & Banfield, 2001) but a genome-wide survey suggests that the potential number is much larger (Katz *et al.*, 2003). The products of these methyltransferase reactions are a methylated product and *S*-adenosylhomocysteine (SAH), which is subsequently hydrolyzed by SAH hydrolase (EC 3.3.1.1) to homocysteine and adenosine. This series of reactions, known as transmethylation, probably occurs in every cell of the body. Homocysteine has several metabolic fates. It may be remethylated back to methio-

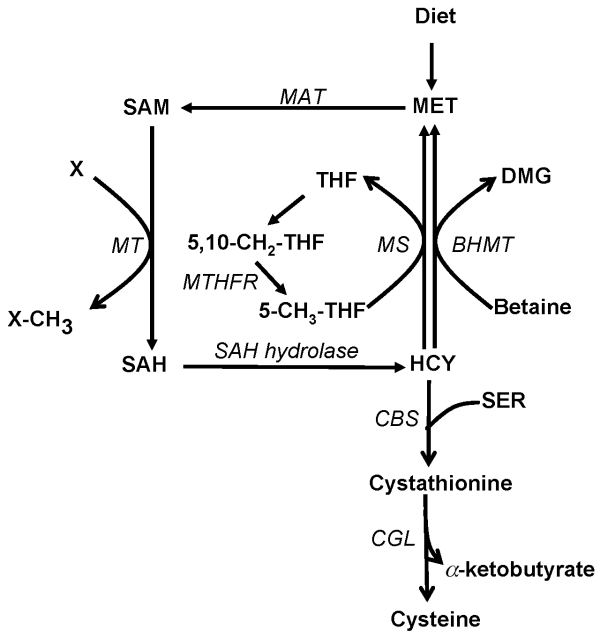

Figure 2. Outline of methionine metabolism.

nine by one of two enzymes. Betaine: homocysteine methyltransferase (BHMT; EC 2.1.1.15) has a rather limited tissue distribution, principally in the liver but, in some species, it occurs in the kidney. Methionine synthase (MS; EC 2.1.1.13) uses a methyl group from the one carbon pool (5-methyltetrahydrofolate, 5-MeTHF) as methyl donor. Methionine synthase is widely distributed and this remethylation pathway is thought to be ubiquitously distributed. Together, transmethylation and remethylation comprise the methionine cycle. This cycle does not accomplish methionine catabolism. This involves the transsulfuration pathway which consists of the enzymes cystathionine β-synthase (CBS; EC 4.2.1.22) and cystathionine γ-lyase (CGL; EC 4.4.1.1). This series of reactions converts homocysteine to cysteine and accounts for the fact that cysteine is not a dietary essential amino acid, provided adequate supplies of methionine are available. The transsulfuration pathway has a limited distribution, being only found in the liver, kidney, small intestine and pancreas. Homocysteine may also move from cells to the blood. The transporter which effects the export of homocysteine from cells has not been identified.

REGULATION OF PLASMA HOMOCYSTEINE LEVEL – THE ROLE OF HOMOCYSTEINE REMOVAL

Most of the early work on the causes of hyperhomocysteinemia focused on impaired homocysteine removal, caused by nutritional or genetic factors or by renal disease. Homocysteine metabolism is highly dependent on vitamin-derived cofactors. MS contains cobalamin as its prosthetic group and derives its methyl group from the folic acid, one-carbon pool. Both of the transsulfuration enzymes (CBS and CGL) contain pyridoxal phosphate as their prosthetic group. Deficiencies of any of these vitamins (Vitamin B_{12}, folic acid and Vitamin B_6) are associated with hyperhomocysteinemia (Selhub *et al.*, 2000). Folic acid deficiency is probably the commonest cause of hyperhomocysteinemia and, in the United States of America, folic acid fortification of staple foodstuffs has decreased mean population tHcy by about 8% (Jacques *et al.*, 1999).

Genetic mutations have long been known to cause hyperhomocysteinemia. In particular, deficiencies of either CBS or methylene tetrahydrofolate reductase (MTHFR; EC 1.1.99.15) can result in homocysteinuria and very severe hyperhomocysteinemia (Kraus, 1998; Goyette *et al.*, 1995). These are very rare inborn errors of metabolism. Recently, attention has focused on the role of a number of genetic polymorphisms which have a more extensive distribution in the general population. In particular, a very common polymorphism of MTHFR (C677T) is homozygous in about 10–16% of many populations (Goyette *et al.*, 1995). The substitution of valine, at position 222, for alanine produces an MTHFR which has an enhanced ability to dissociate

into its monomers and lose its FAD cofactor. This loss of activity is particularly pronounced at low concentrations of 5-MeTHF (Yamada *et al.*, 2001). There is, therefore, an interesting relationship between this MTHFR polymorphism and folic acid status. Individuals with the C677T polymorphism have higher tHcy when plasma folate levels are low (Jacques *et al.,* 1996). The C677T genotype predisposes to an increased risk of neural tube defects (van der Put *et al.*, 1995) and of cardiovascular disease (Klerk *et al.,* 2002).

End-stage renal disease is, almost invariably, associated with moderate hyperhomocysteinemia and this has been implicated in the increased risk of cardiovascular disease of these patients (Bostom *et al.*, 1995a). The metabolic basis for this hyperhomocysteinemia has not been entirely resolved. We have shown that rat kidneys metabolize homocysteine *in vivo* (Bostom *et al.*, 1995b) but this has not been confirmed in humans (van Guldener *et al.*, 1998). However, kinetic studies by Guttormsen *et al.* (1997) have clearly demonstrated that the metabolic removal of a homocysteine load is greatly impaired in end-stage renal disease and that this likely occurs as a result of decreased transsulfuration.

REGULATION OF PLASMA HOMOCYSTEINE LEVEL — METHYLATION DEMAND; THE ROLE OF HOMOCYSTEINE PRODUCTION

It is our thesis that we must address the reactions that produce homocysteine as well as those that remove it. We emphasize the importance of flux through the methyltransferase reactions in producing homocysteine and, therefore, in affecting plasma homocysteine levels. This is evident from studies with high intakes of methionine. Hyperhomocysteinemia occurs after methionine loading in humans (Silberberg *et al.*, 1997) and in rats (Miller *et al.*, 1994). This situation is characterized by a high rate of methionine catabolism, requiring increased flux through the overflow enzyme, glycine *N*-methyltransferase (EC 2.1.1.20), at greater rates than homocysteine can be removed by transsulfuration and remethylation. The concept of methylation demand, however, invokes situations that occur at adequate, not excessive, methionine intakes. We advance the concept that high-flux methylation of a number of substrates, both physiological and pharmacological, plays a crucial role in determining the plasma homocysteine level. In essence, we advocate the view that we must take into account both the reactions that produce homocysteine and those that remove it for a complete understanding of homocysteine homeostasis.

METHYLATION OF EXOGENOUS COMPOUNDS: L-DOPA

The first demonstration of the phenomenon of methylation demand came from studies on patients with Parkinson's disease undergoing treatment with L-DOPA (L-3,4-dihydroxyphenylalanine). This disease is characterized by a marked depletion of nigro-striatal dopaminergic neurons, resulting in deficiencies of dopamine in the basal ganglia and of melanin in the substantia nigra (Cheng *et al.*, 1997). Treatment often involves the administration of L-DOPA, either alone or in combination with a peripheral decarboxylase inhibitor. L-DOPA enters the brain and is decarboxylated there, which relieves the deficiency (Miller *et al.*, 1997).

Plasma homocysteine levels in Parkinson's patients undergoing treatment with L-DOPA are about 50% higher than in healthy controls (Allain *et al.*, 1995; Muller *et al.*, 2001). L-DOPA is a substrate for catechol-*O*-methyltransferase (COMT; EC 2.1.1.6) which catalyzes the SAM-dependent methylation of aromatic hydroxyl groups (Cheng *et al.*, 1997;

Palma *et al.*, 2003). During L-DOPA therapy, a wasteful methylation by COMT occurs both centrally and peripherally (Miller *et al.*, 1997; Palma *et al.*, 2003). This "wasteful" metabolism of L-DOPA through methylation means that quite high doses (several grams) needs to be administered. That methylation of L-DOPA *via* COMT is responsible for the elevated homocysteine concentrations has been demonstrated by elegant experiments on rats by Miller *et al.* (1997). Rats that received L-DOPA exhibited elevated tHcy and decreased tissue SAM concentrations. These responses were eliminated or attenuated by pre-treatment with a COMT inhibitor.

METHYLATION OF ENDOGENOUS SUBSTRATES: SYNTHESIS OF CREATINE AND PHOSPHATIDYLCHOLINE

Creatine synthesis

Creatine synthesis is considered to be the major user of methyl groups from SAM. Mudd and Poole (1975) and Mudd *et al.* (1980) have calculated that this process utilizes more SAM than all of the other physiological methyltransferases combined, accounting for about 75% of homocysteine formation. Creatine synthesis is a simple pathway, involving only two enzymes, L-arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1) and guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2). High activities of AGAT are found in the kidney and of GAMT in the liver and it is suggested that, to some degree, creatine synthesis is an inter-organ pathway (Fig. 3). Creatine is known to exert feedback control on AGAT at the pre-translational level (McGuire *et al.*, 1984). GAMT appears to be regulated by GAA availability. We carried out a series of experiments in rats to examine the proposition that modulation of methylation demand by dietary provision of creatine or GAA could affect homocysteine metabolism (Stead *et al.*, 2001). Our hypothesis was that dietary GAA would impose a methylation demand that would increase tHcy whereas dietary creatine would result in a decreased methylation demand and a decrease in tHcy.

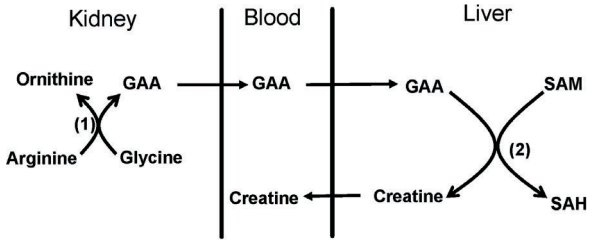

Figure 3. Interorgan synthesis of creatine.

1, L-arginine:glycine amidinotransferase; 2, guanidinoacetate *N*-methyltransferase.

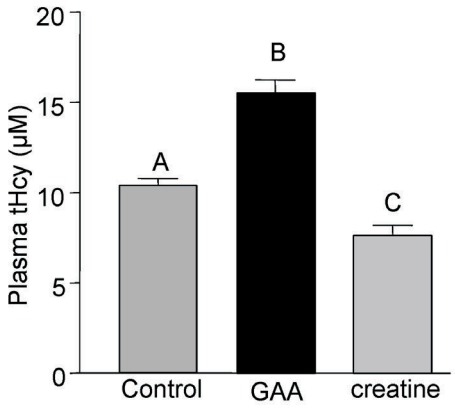

Figure 4. Plasma homocysteine levels in rats fed dietary creatine or guanidinoacetate (GAA).

$P < 0.05$ was taken to indicate statistical significance. Bars with different letters are different from each other. From Stead *et al.* (2001), with permission.

The results of these experiments are shown in Fig. 4. Creatine ingestion decreased tHcy by about 25% and GAA ingestion increased it by about 50%. There was no change in plasma methionine. Both creatine and GAA ingestion caused very large decreases (about 80%) in renal AGAT activity. We have since shown that creatine ingestion markedly decreases plasma GAA levels and its arterio-venous difference across the kidney. These results indicate that creatine synthesis, in rats, is an im-

portant determinant of homocysteine metabolism and of plasma homocysteine levels.

Phosphatidylcholine synthesis

We have recently examined, in collaboration with Dr. Dennis Vance, the role of phosphatidylcholine (PC) synthesis *via* phosphatidylethanolamine *N*-methyltransferase (PEMT; EC 2.1.1.17) in determining plasma homocysteine levels. Figure 5 shows the pathways of PC synthesis in liver. PEMT is a liver-specific enzyme that uses three successive SAM-dependent methylations to convert phosphatidylethanolamine to PC. Hepatic PC may also be produced, beginning with choline, by means of the Kennedy pathway which does not utilize SAM. The PEMT pathway accounts for about 30% of hepatic PC synthesis (Reo *et al.*, 2002). In view of the appreciable quantities of PC synthesized in the liver and the utilization of three SAM molecules for every molecule of PC synthesized *via* the PEMT reaction, we examined the role played by this reaction in homocysteine metabolism.

We examined this question, using the *Pemt*$^{-/-}$ mouse. We also employed hepatocytes derived from these mice and McArdle RH7777 (rat hepatoma) cells in which PEMT had been overexpressed (Noga *et al.*, 2003). Figure 6 shows that chow-fed *Pemt*$^{-/-}$ mice had appreciably lower (about 50%) tHcy compared to wild-type, *Pemt*$^{+/+}$, controls. This remarkable decrease in plasma homocysteine could not be accounted for by changes in the hepatic enzymes of homocysteine metabolism. Primary hepatocytes from *Pemt*$^{-/-}$ mice, cultured in Dulbecco's medium, produced only about 50% as much homocysteine as hepatocytes from *Pemt*$^{+/+}$ mice. Furthermore, expression of PEMT in McArdle cells, which normally have very low activity of this enzyme, greatly increased homocysteine production by these cells (Noga *et al.*, 2003). These results clearly implicate flux through PEMT as a major determinant of plasma

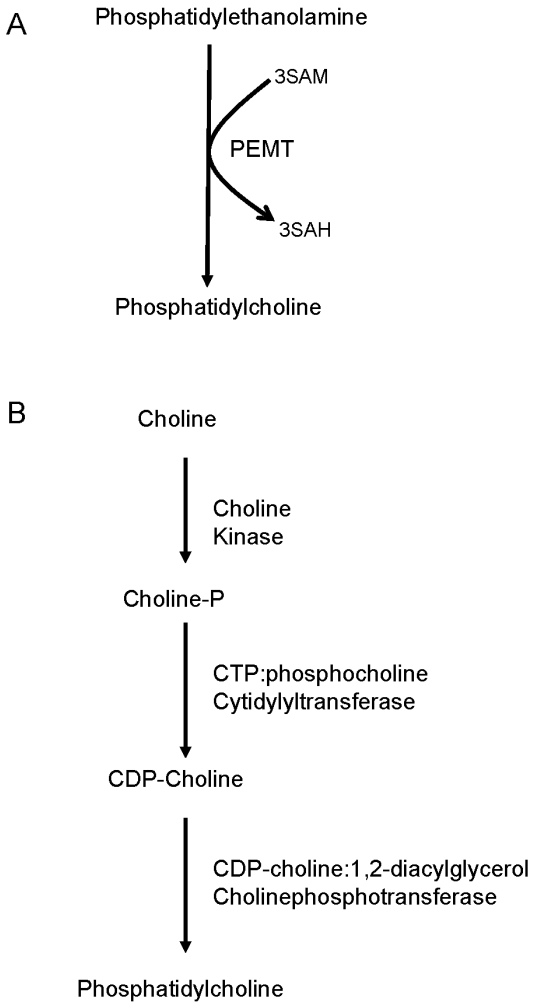

Figure 5. Pathways for the hepatic synthesis of phosphatidylcholine.

A. Phosphatidylcholine synthesis from phosphatidylethanolamine; B. Phosphatidylcholine synthesis from choline (Kennedy pathway).

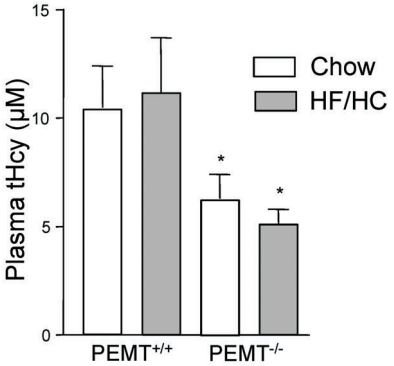

Figure 6. Plasma homocysteine levels in *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice that were fed chow or a high fat/high cholesterol diet (HF/HC).

*indicates $P < 0.001$ for *Pemt*$^{-/-}$ compared to *Pemt*$^{+/+}$ mice. From Noga *et al.* (2003), with permission.

homocysteine levels. Since PEMT is confined to the liver, these results, together with those on creatine synthesis, point to liver metabolism (specifically the major hepatic methyltransferases) as playing a dominant role in determining plasma homocysteine levels.

CONCLUSION

Elevated plasma homocysteine is an important risk factor for diseases such as cardiovascular disease and Alzheimer's disease. It is, therefore, important to understand the factors that affect homocysteine metabolism and determine homocysteine levels. It is clear, from our results, that it is necessary to consider the reactions that produce homocysteine in addition to the pathways for its removal. Experiments in rodents identify two major hepatic methyltransferases, GAMT in creatine synthesis and PEMT in PC synthesis, as major determinants of plasma homocysteine. These insights into the production of homocysteine may provide us with approaches that may be useful for the therapeutic lowering of plasma homocysteine levels.

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