Increase in Plasma Homocysteine Associated with Parallel Increases in Plasma S-Adenosylhomocysteine and Lymphocyte DNA Hypomethylation

S-Adenosylmethionine and S-adenosylhomocysteine (SAH), as the substrate and product of essential cellular methyltransferase reactions, are important metabolic indicators of cellular methylation status. Chronic elevation of SAH, secondary to the homocysteine-mediated reversal of the SAH hydrolase reaction, reduces methylation of DNA, RNA, proteins, and phospholipids. High affinity binding of SAH to the active site of cellular methyltransferases results in product inhibition of the enzyme. Using a sensitive new high pressure liquid chromatography method with coulometric electrochemical detection, plasma SAH levels in healthy young women were found to increase linearly with mild elevation in homocysteine levels \( (r = 0.73; p < 0.001) \); however, S-adenosylmethionine levels were not affected. Plasma SAH levels were positively correlated with intracellular lymphocyte SAH levels \( (r = 0.81; p < 0.001) \) and also with lymphocyte DNA hypomethylation \( (r = 0.74, p < 0.001) \). These results suggest that chronic elevation in plasma homocysteine levels, such as those associated with nutritional deficiencies or genetic polymorphisms in the folate pathway, may have an indirect and negative effect on cellular methylation reactions through a concomitant increase in intracellular SAH levels.

An elevation in plasma homocysteine is a sensitive but nonspecific biomarker for an imbalance in the integrated pathways of one-carbon metabolism \( (1, 2) \). Chronic nutritional deficiencies in folate, choline, methionine, vitamin B₁₂, and/or vitamin B₆ can perturb the complex regulatory network that maintains normal one-carbon metabolism and homocysteine homeostasis \( (3–7) \). Genetic polymorphisms in these pathways can act synergistically with nutritional deficiencies to accelerate the metabolic pathology associated with chronic disease states \( (8) \). Although several hypotheses have been proposed to explain the association between hyperhomocysteinemia and the thrombotic/atherosclerotic process occurring with occlusive cardiovascular disease, as yet none has been definitive \( (9–12) \). Similarly, increases in plasma homocysteine concentrations have been associated with increased risk of certain birth defects \( (13–16) \), but the underlying mechanism remains elusive. A major unanswered question is whether direct cellular toxicity of homocysteine is causally involved in pathogenesis or whether homocysteinemia is simply a passive and indirect indicator of a more complex mechanism.

Homocysteine is derived solely from methionine metabolism and is significantly recycled to conserve sufficient methionine for protein and S-adenosylmethionine synthesis. The interactive and interdependent pathways of the methionine/homocysteine cycle are diagrammed in Fig. 1 to emphasize the indirect effects of pathway perturbations on cellular methyltransferase reactions. The metabolic generation of homocysteine from methionine is initiated by the ATP-dependent transfer of adenosine to methionine via methionine adenosyltransferase. The product, S-adenosylmethionine (SAM), \(^1 \), is a priority for one-carbon metabolism because it is the methyl donor for most cellular methyltransferase reactions. In addition to DNA methylation, SAM-dependent methyltransferase activity is essential for hundreds of other cellular methylation reactions including synthesis of creatine in the liver, membrane phosphatidylcholine synthesis, central nervous system neurotransmitter synthesis, methylation/detoxification, and RNA and protein methylation \( (17) \). After transfer of the methyl group, SAM is converted to S-adenosylhomocysteine (SAH) within the active site of the methyltransferase enzyme. Because most methyltransferases bind SAH with higher affinity than SAM, they are subject to potent product inhibition by SAH \( (18) \). Thus, the efficiency of methyltransferase reactions is absolutely dependent on efficient product removal of SAH. This is effectively accomplished by SAH hydrolase (SAHH), an enzyme that appears to act in close proximity to the methyltransferases, at least in the nucleus \( (19) \). The crystal structure of SAHH has been recently reported, and interestingly, the polypeptide folding pattern at the catalytic domain of SAHH is almost identical to that reported for the DNA methyltransferases and suggests that SAH molecules can travel easily between the catalytic pockets of the two enzymes \( (20) \). This binding site similarity further supports an important role for excess SAH in the regulation of methyltransferase activity \( (20) \).

\(^1 \) The abbreviations used are: SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SAHH, SAH hydrolase; HPLC, high performance liquid chromatography; tHcy, total homocysteine; THF, tetrahydrofolate; DMG, dimethylglycine.
An overview of one-carbon metabolism with emphasis on the reversible SAH hydrolase reaction (3). The hydrolysis of SAH is dependent on product removal of homocysteine and adenosine. In the absence of efficient product removal, SAH accumulation can inhibit methyltransferase activity by high affinity binding to the enzyme active site.

Under normal physiologic conditions, SAH is hydrolyzed by SAHH to adenosine and homocysteine. It is important to note, however, that this reaction is readily reversible with equilibrium dynamics that strongly favor SAH synthesis rather than hydrolysis. In fact, the only reason that this reaction proceeds in the hydrolytic direction is efficient product removal (21). Thus, metabolic perturbations that interfere with the efficient removal of homocysteine and adenosine will lead to an increase in SAH (22). The existence of multiple routes of removal for both these metabolites is consistent with the necessity for efficient product removal to avoid SAH accumulation and the potentially negative consequences of methyltransferase inhibition (1, 23). Homocysteine can be methylated to regenerate methionine in all cells by the folate/B12-dependent methionine synthase reaction and additionally by the betaine-homocysteine methyltransferase reaction in liver and kidney of humans (1). A third route of homocysteine removal is the irreversible pyridoxal phosphate-dependent transsulfuration pathway in which cystathionine β synthase and lyase reactions permanently remove homocysteine from the methionine cycle. Adenosine can be efficiently removed by either the adenosine deaminase reaction or the adenosine kinase reaction. Experimental studies have shown that analog inhibition of these pathways or genetic deficiencies in these enzymes results in SAH accumulation and potent inhibition of methyltransferases (24–26).

In the present report, using a sensitive new method for measuring plasma concentrations of SAM and SAH, we show for the first time that moderate elevation in plasma total homocysteine concentration is positively associated with parallel increases in plasma SAH concentrations and lymphocyte DNA hypomethylation. These data support an indirect mechanism for homocysteine pathogenicity secondary to SAH-mediated inhibition of the DNA methyltransferase. The disruption of the heritable methylation patterns in DNA can lead to alterations in chromatin structure and alterations in gene expression that can promote chronic disease states (27–30).

MATERIALS AND METHODS

Reagents—SAM, SAH, trichloroacetic acid, sodium phosphate monobasic, monohydrate, and 1-heptanesulfonic acid were obtained from Sigma. HPLC grade methanol was purchased from J.T. Baker Inc. (Phillipsburg, NJ). Deionized HPLC-grade water for HPLC was prepared by passage through a Syroban/Barusted NANOpure II filtration system (Boston, MA) and subsequent passage through a C18 Sep-Pak cartridges (Millipore Corp., Milford, MA).

Subjects and Blood Collection—Participants were 58 healthy adult females with a mean age of 37.2 years (range, 19–53 years) who had participated in a previous clinical study (15). Fasting blood samples were collected into EDTA-Vacutainer tubes, immediately chilled on ice, and centrifuged at 400 × g for 15 min at 4 °C. Aliquots of the plasma layer were transferred into multiple cryostat tubes and stored at −20 °C until analysis. Individual aliquots were thawed for determination of plasma homocysteine, methionine, SAM, and SAH. DNA was extracted from the cell pellet using standard chloroform/phenol methodology (31). In a subset of women, mononuclear cells were immediately isolated by carefully layering whole blood onto an equal volume of Histopaque® 1077 (Sigma) at room temperature and centrifuging at 400 × g for 30 min. Mononuclear cells were recovered from the interface and washed several times as described by the manufacturer, and aliquots of approximately 10⁶ cells were homogenized in 200 μl of phosphate-buffered saline. The homogenate was centrifuged at 18,000 × g for 1 min, and the supernatant was stored at −80 °C until HPLC analysis.

Sample Preparation—For determination of total homocysteine (tHcy) and methionine, 50 μl of freshly prepared 1.43 M sodium borohydride solution containing 1.5 μM EDTA, 66 mM NaOH, and 10 μl of n-amylic alcohol were added to 200 μl of plasma or cell homogenate. After gentle mixing, the solution was incubated in 40 °C water bath for 30 min with gentle shaking. To precipitate proteins, 250 μl of ice-cold 10% meta-phosphoric acid was added, and the sample was incubated for 10 min on ice. After centrifugation at 18,000 × g for 15 min at 4 °C, the supernatant was filtered through a 0.2-μm filter (PGC Scientific, Frederick, MD), and a 20-μl aliquot was injected into the HPLC system. For determination of SAM and SAH, 40 μl of 40% trichloroacetic acid were added to 200 μl of plasma or cell extract to precipitate protein, mixed well, and incubated on ice for 30 min. After centrifugation for 15 min at 18,000 × g at 4 °C, supernatants containing SAM and SAH were passed through a 0.2-μm filter and 20 μl was injected into the HPLC system.

HPLC Chromatography—The elution of homocysteine and methionine utilized a different mobile phase than that used for elution of SAM and SAH; however, both analyses were accomplished using HPLC with a Shimadzu solvent delivery system (ESA model 580) and a reverse phase C18 column (5 μm; 4.6 × 150 mm, MCM, Inc., Tokyo, Japan) obtained from ESA, Inc. (Chelmsford, MA). A 20-μl aliquot of plasma or cell extract was directly injected onto the column using a Beckman autosampler (model 507E). To assure standardization between sample runs, calibration standards and reference plasma samples were interspersed at intervals during each run. For elution of homocysteine and methionine, the mobile phase consisted of 50 mM sodium phosphate monobasic monohydrate, 1.0 mM ion-pairing reagent octane sulfonic acid, 2% acetonitrile (v/v) adjusted to pH 2.7 with 85% phosphoric acid, with isocratic elution at ambient temperature at a flow rate of 1.0 ml/min and a pressure of 120–140 kgf/cm² (1800–2100 psi). For elution of SAM and SAH, the mobile phase consisted of 50 mM sodium phosphate monobasic, monohydrate, 1.0 mM 1-heptanesulfonic acid, 7.5% (v/v) methanol adjusted to pH 3.4 with 85% phosphoric acid, with isocratic elution at ambient temperature at a flow rate of 1.0 ml/min.
TABLE I
Plasma levels of homocysteine, methionine, SAH, and SAM in individuals with normal (5.8–8.7 μM) and elevated (9.3–16.5 μM) levels of plasma homocysteine

|                  | Normal tHcy | Elevated tHcy |
|------------------|-------------|---------------|
| mean ± S.D., n = 28 | mean ± S.D., n = 30 |
| Homocysteine (μM) | 7.26 ± 1.11 | 12.30 ± 1.82* |
| Methionine (μM)   | 38.30 ± 9.71 | 28.80 ± 6.25* |
| Homocysteine/methionine ratio | 0.20 ± 0.05 | 0.50 ± 0.17* |
| SAM (μM)          | 79.90 ± 8.81 | 76.41 ± 6.13  |
| SAH (nm)          | 20.00 ± 5.55 | 40.10 ± 12.5* |
| SAM/SAH ratio     | 4.43 ± 1.48 | 2.40 ± 1.28*  |

*p < 0.001 as compared to group with normal homocysteine.

and a pressure of 100–110 kgf/cm² (1500–1800 psi). tHcy, methionine, SAM, and SAH were quantified using a model 5200A Coul彻om II electrochemical detector (ESA, Inc.) equipped with a dual analytical cell (model 5010) and a guard cell (model 5020). Methodologic details have been described previously (32).

**Lymphocyte Global DNA Methylation Using Cytosine Extension Assay**—Assessment of lymphocyte DNA methylation was accomplished using the cytosine extension assay previously described in detail (33). Briefly, ~1 μg of genomic DNA was digested for 16–18 h with 20 units of HpaII according to manufacturer's protocol (New England Biolabs, Beverly, MA). A second DNA aliquot served as background control and was similarly incubated without addition of restriction enzyme. The single nucleotide extension reaction was performed in a 25-μl reaction mixture containing 0.5 μg of DNA, 1× polymerase chain reaction buffer II, 1.0 mM MgCl₂, 0.25 units of AmpliTaq DNA polymerase (Perkin-Elmer), and 0.1 μl of [³H]dCTP (57.4 Ci/mmol, NEN Life Science Products), incubated at 36 °C for 1 h, and then placed on ice. Duplicate 10-μl aliquots from each reaction were applied onto Whatman DE-81 ion exchange filters and washed three times with 0.5 M sodium phosphate buffer (pH 7.0) at room temperature. Filters were dried and processed for scintillation counting in 10 ml of Ultima Gold (Packard Bioscience Co., Meriden, CT). Background radiolabel incorporation in untreated samples is subtracted from enzyme-treated samples, and the results are expressed as relative [³H]dCTP incorporation/0.5 μg DNA.

**Statistics**—Data are presented as the means ± S.D. Statistical differences between means were calculated using the Student’s t test and Sigmasstat software (Jandel Scientific, San Rafael, CA).

**RESULTS**

**Mean Plasma Concentrations of Methionine, SAM, and SAH in Women with Normal and Elevated tHcy Concentrations**—In Table I, mean values for plasma homocysteine, methionine, SAM, and SAH are shown as a function of fasting plasma tHcy levels in the 58 participants. The women were stratified by tHcy based on previously published normal ranges for adult females (34, 35). In this cohort, women with tHcy ranging from 5.8 to 8.7 μM (mean, 7.26 ± 1.1) were designated to be within the “normal” range of tHcy and women with tHcy ranging from 9.3 to 16.5 μM (mean, 12.3 ± 1.82) were designated as having “elevated” tHcy concentrations. Among the women within the normal range of tHcy, the mean plasma SAM concentration was 79.9 ± 8.81 μM, the mean SAH concentration was 20.0 ± 5.55 nm, and the SAM/SAH ratio was 4.43 ± 1.48. Among the women with elevated tHcy, plasma methionine concentrations were significantly decreased, and the ratio of tHcy/methionine was significantly increased relative to the women within the normal tHcy range (p < 0.001). Elevated plasma tHcy was not associated with an alteration in SAM levels, but SAH levels were increased 2-fold relative to women with normal tHcy, and the SAM/SAH ratio was decreased by one-half (p < 0.001).

**Relationship between Plasma Homocysteine and Plasma Levels of SAM, SAH, and Methionine**—Fig. 2A is a plot of the individual values of plasma tHcy and the corresponding plasma methionine values. Fig. 2B is a similar plot showing the relationship between plasma tHcy and SAH for each participant, and Fig. 2C shows the relationship between plasma tHcy and SAM. A modest but significant negative correlation was found between plasma tHcy and methionine (r = 0.50; p < 0.01). Increasing concentrations of plasma tHcy were strongly associated with increased concentrations of plasma SAH (r = 0.73; p < 0.001), whereas there was no apparent relationship between plasma SAM and tHcy. A strong negative correlation was found between tHcy and the ratio of SAM/SAH (r = 0.73, p < 0.01; data not shown); the decrease in SAM/SAH ratio was due to the increase in SAH in all cases.

**Relationship between Plasma SAH and Intracellular Lymphocyte SAH**—Intracellular SAH concentration was determined in extracts of fresh lymphocytes isolated from a subset of the participants. Fig. 3 is a plot of individual plasma SAH concentration and the corresponding intracellular lymphocyte SAH concentration for each individual. Regression analysis
indicated a significant positive correlation between plasma and intracellular SAH concentrations \((r = 0.81; p < 0.001)\).

**Relationship between Mean Plasma SAM and SAH Concentrations and Lymphocyte DNA Methylation**—The plasma SAH concentrations and relative levels of DNA methylation were compared between the women with normal levels of plasma tHcy and the women with elevated plasma tHcy. The level of DNA hypomethylation is defined as the extent of \(^{3}H\)dCTP incorporation into DNA after treatment with the methyl-sensitive restriction enzyme, \(Hpa\II\), that cuts DNA leaving a guanine overhang at unmethylated recognition sites (33). An increase in radiolabel incorporation reflects the increased number of unmethylated cytosines in DNA. In Fig. 4, a plot of the individual values of plasma SAM and SAH, respectively, are correlated with the extent of lymphocyte DNA hypomethylation. Regression analysis indicated a significant positive association between SAH and DNA hypomethylation \((r = 0.74, p < 0.001)\); however, there was no apparent correlation between DNA hypomethylation and SAM values. In Fig. 5, the mean SAH values are shown to be increased 2-fold, and DNA hypomethylation increased 2.6-fold in women with elevated tHcy (range, 9.3–16.6 \(\mu M\)) relative to women with normal tHcy (range, 5.8–8.7 \(\mu M\)).

**DISCUSSION**

In recent years, a decrease in the ratio of SAM/SAH has been used frequently as a predictor of reduced cellular methylation. In these studies, the decrease in SAM has been emphasized as a limiting cofactor for methyltransferase activity and the major effector of the reduced ratio (36–38). However, earlier studies of alterations in SAM/SAH using nitrous oxide, SAHH inhibition, or cell lines from genetically deficient fibroblasts clearly demonstrated that an increase in SAH, with or without a decrease in SAM, was the more important variable in predicting methyltransferase inhibition and a decrease in cellular methylation (24–26, 39, 40). For example, a decrease in SAM/SAH ratio in the presence of an increase or no change in SAM, was reproducibly associated with hypomethylation and decreased methyltransferase activity (22, 23, 26, 42). It is possible to induce an independent decrease in SAM without a concomitant increase in SAH by genetic or chemical inhibition of methionine adenosyltransferase. Under these conditions, SAM becomes severely depleted below the \(K_m\) of most methyltransferases and has resulted in DNA hypomethylation (43) and central nervous system demyelination (44). It is questionable, however, whether physiologic decreases in SAM, such as those induced by nutritional deficiencies, are causally related to cellular hypomethylation. It is important to recognize that the use of the SAM/SAH ratio as a predictor of altered cellular methylation can, in fact, be quite misleading and that evaluation of alterations in individual components may be more informative. For example, it has been shown that identical decreases in SAM/SAH ratio are conditionally associated with reduced methylation capacity depending on the absolute value of SAH (45).2 Consistent with this concept, the results presented here suggest that an increase in SAH, secondary to an increase in homocysteine, is more strongly correlated with DNA hypomethylation than are alterations in

\(^2\) S. J. James, personal observations.
SAM, at least within physiologic ranges of homocysteine.

Cellular methyltransferases that have been shown experimentally to be inhibited by SAH include catecholamine-O-methyltransferase (39), phosphatidylethanolamine methyltransferase (46), histone methyltransferase (18), DNA methyltransferase (18, 26, 47), tRNA and mRNA methyltransferases (48, 49), acetylserotonin methyltransferase (50), and histamine N-methyltransferase (51). The functional consequences of decreased cellular methylation are significant and include central nervous system demyelination (52, 53), reduced neurotransmitter synthesis (39, 50), decreased chemotaxis and macrophage phagocytosis (54, 55), altered membrane phospholipid composition and membrane fluidity (56, 58), altered gene expression (23, 59, 60), and cell differentiation (61, 62). It is likely that the $K_c$ for SAH varies with different cellular methyltransferases and also varies according to tissue priorities and subcellular methyltransferase distribution (63). Tissue levels of SAH reflect the balance between rate of synthesis and the direction of the reversible SAH hydrolase reaction (18). Intraacellular SAH can be exported across the plasma membrane against a concentration gradient and appears to be carrier-mediated and largely unidirectional in lymphocytes (63). An increase in SAH has a positive regulatory influence on cystathionine $\beta$ synthase (64) and methylenetetrahydrofolate reductase activities (65), and SAH has been shown to down-regulate rat liver betaine homocysteine methyltransferase and porcine kidney methionine synthase (66). Taken together, experimental evidence supports a regulatory role for SAH in maintaining normal one-carbon metabolism.

Tissue-specific gene expression depends on the stable inheritance of DNA methylation patterns established during embryogenesis. In differentiated cells, genes are silenced by promoter region methylation in a tissue-specific manner. Disruption of the nonrandom DNA methylation patterns can lead to inappropriate gene expression and promotion of chronic disease (27, 28, 52). Although most cells express genes required for the methionine remethylation, not all the cell expresses genes for the transsulfuration pathway. For example, spleen, adrenal, lung, testes, and heart tissue exhibit negligible cystathionine $\beta$ synthase activity (67). Thus, tissues lacking appreciable transsulfuration activity might be expected to be most sensitive to increases in SAH and effects on cellular methylation. Of related interest, inactivating mutations in the adenosine deaminase gene lead to severe combined immune deficiency and profound lymphocytopenia. Although the adenosine deaminase enzyme is ubiquitous in cells, the unique sensitivity of lymphocytes may be partly explained by the lack of transsulfuration pathway and increased sensitivity to SAH. Consistent with this notion, resting lymphocytes have been shown to turnover SAM at a rate 3–5 times higher than that estimated for most non-hepatic tissues (68). Further, lymphocyte DNA hypomethylation was recently documented in women undergoing controlled folate depletion (69, 70). Taken together, these observations suggest that global hypomethylation in lymphocyte DNA may be an early biomarker of abnormal methylation in other tissues. Further, the correlation between plasma homocysteine and DNA hypomethylation suggests an indirect mechanism for homocysteine-related disease pathology.

In the present report, the increase in plasma total homocysteine was highly correlated with a parallel increase in SAH; however, no apparent association with SAM was observed. The increase in plasma SAH was also associated with a progressive increase in lymphocyte DNA hypomethylation. It is important to emphasize, however, that the relationship between tissue levels of SAM and SAH and plasma levels of these metabolites is complex and that the tissue-specific origins of plasma SAM and SAH are not known. Interestingly, a modest but significant decrease in plasma methionine levels was associated with the increase in homocysteine. The ratio of the homocysteine to methionine may provide a sensitive clinical biomarker for agents or conditions that compromise methionine synthase activity. For example, a decrease in methionine is consistent with the reduction in methionine synthase activity because of reduced availability of 5-methyltetrahydrofolate. Nutritional folate deficiency has been associated with a decrease in methionine levels (72) and would be expected to decrease the methionine/homocysteine ratio. This ratio may also be useful in the differential diagnosis of genetic aberrations in cystathionine $\beta$ synthase and MTHFR genes. Both conditions are associated with elevations in tHcy but have opposite effects on methionine. Thus, the ratio would be expected to increase with cystathionine $\beta$ synthase deficiency and to decrease with MTHFR deficiency.

The lack of correlation between SAM and DNA hypomethylation would suggest that SAM is not a limiting factor for the DNA methyltransferase, at least within physiologic ranges. However, low levels of SAM are clearly associated with up-regulation of the MTHFR enzyme to divert 5,10-methyltetrahydrofolate toward methionine synthase and its own resynthesis (73). Therefore, rather than an effect on DNA methylation, low SAM levels may have a greater regulatory impact on DNA synthesis by diverting 5,10-methylenetetrahydrofolate away from de novo thymidine and purine synthesis. The ability to measure plasma levels of SAM and SAH sensitively and reproducibly should provide new insights into the disregulation of one carbon metabolism in humans.

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