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Interaction between retinoids and methyl group metabolism

Marlies K. Ozias
Iowa State University

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Interaction between retinoids and methyl group metabolism

by

Marlies K. Ozias

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Program of Study Committee:
Kevin L. Schalinske, Co-Major Professor
Suzanne Hendrich, Co-Major Professor
Mark R. Ackermann
Manju B. Reddy

Iowa State University

Ames, Iowa

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Graduate College
Iowa State University

This is to certify that the master's thesis of
Marlies K. Ozias
has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

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LIST OF ABBREVIATIONS

- α -KB, α -ketobutyrate
- ATRA, all-*trans*-retinoic acid
- BHMT, betaine:homocysteine methyltransferase
- CAD, coronary artery disease
- CBS, cystathionine β -synthase
- CRA, 13-*cis*-retinoic acid
- CYP1A1, cytochrome P4501A1
- DFE, dietary folate equivalent
- DHF, dihydrofolate
- DMG, dimethylglycine
- GNMT, glycine *N*-methyltransferase
- GSH, glutathione
- HCC, hepatocellular carcinoma
- MS, methionine synthase
- MTHFR, 5,10-methylenetetrahydrofolate reductase
- NTDs, neural tube defects
- PAH, polycyclic aromatic hydrocarbon
- RDA, recommended dietary allowance
- SAH, S-adenosylhomocysteine
- SAM, S-adenosylmethionine
- THF, tetrahydrofolate

ABSTRACT

Methyl group and folate-dependent one-carbon metabolism play an integral role in health and disease. The donation of carbon in the production of nucleic acids from folate coenzymes and the methylation of biological compounds via transmethylation metabolism is important for maintenance of cells and tissues. Disruption of these tightly regulated pathways can result from numerous factors, including genetic polymorphisms of key enzymes, nutritional deficiencies, hormonal imbalances, or drug-nutrient interactions. Glycine *N*-methyltransferase (GNMT) is a key protein that regulates the supply of methyl groups for S-adenosylmethionine-dependent transmethylation reactions. We have shown that retinoid administration increases GNMT activity and protein abundance, thereby leading to the loss of methyl groups. Previous studies used pharmacological doses (30 $\mu\text{mol/kg}$ BW) of various retinoids administered daily for a total of 10 d. Here, we examined the dose- and time-dependent relationship between all-*trans*-retinoic acid (ATRA) administration and induction of GNMT, as well as determining additional indices of methyl group, homocysteine, and folate metabolism. For the dose-response study, rats were given either 0, 1, 5, 10, 15 or 30 μmol ATRA/kg BW for 10 d. For the time-course study, rats received 30 μmol ATRA/kg BW for 0, 1, 2, 4, or 8 d. A significant increase ($P = 0.009$) in GNMT activity (105%) was observed with doses as low as 5 $\mu\text{mol/kg}$ BW, whereas maximal induction (231%) of GNMT activity was achieved at 30 $\mu\text{mol/kg}$ BW. Induction of hepatic GNMT by ATRA was rapid, exhibiting a 31% increase following a single dose (1 d) and achieving maximal induction (95%) after 4 d. Plasma methionine and homocysteine concentrations were decreased 42 and 53%, respectively, in ATRA-treated rats compared to control values. In support of this finding, the hepatic activity of methionine synthase, the folate-dependent enzyme required for homocysteine remethylation, was elevated 40% in ATRA-treated rats. This work demonstrates that ATRA administration exerts a rapid effect on hepatic methyl group, folate, and homocysteine metabolism at doses that are within the therapeutic range used by humans.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Complex pathways work efficiently in mammals unless affected by environmental factors or predisposed conditions. Perturbations from pharmaceuticals can disturb this otherwise finely tuned system and have a detrimental effect on overall health. Additionally, nutritional deficiencies and hormonal disturbances to metabolism can disturb a predetermined genetic condition, putting a person at risk for disease. Metabolic pathways are complex and the disruption in the performance of one pathway can have detrimental consequences in complementary pathways. For example, there are many aspects of folate-dependent one-carbon metabolism that can be altered when its cycle is disturbed. The folate cycle donates carbon groups for the synthesis of purines and pyrimidines, the essential components of DNA. Therefore, disruption of folate metabolism can affect DNA synthesis and result in megaloblastic anemia. Many mechanisms are present to control the folate pathway, however disturbances to the interrelated methionine cycle can ultimately affect the synthesis of DNA. The methionine cycle, also known as methyl group metabolism, functions to donate methyl groups in the synthesis of essential compounds like neurotransmitters, phospholipids, creatine, and regulate gene expression. A disturbance of these pathways by nutrient insufficiencies or environmental conditions can result in diseased states including cardiovascular disease and cancer.

Vitamin A is a fat-soluble vitamin that is important in growth and overall health in mammals. Vitamin A is involved in cell differentiation and is used pharmacologically to treat skin disorders such as acne and psoriasis, as well as the treatment of acute promyelocytic leukemia. Although the therapeutic use of retinoids is beneficial, retinoids in high concentration are toxic and can produce damaging side effects; therefore patients are thoroughly examined for side effects before and during the course of treatment.

Vitamin A and its derivatives have the ability to disrupt folate-dependent one-carbon and methyl group metabolism. Research has established a link between retinoids and the modulation of one-carbon metabolism. Recent studies have demonstrated that the most

biologically active retinoid, all-*trans*-retinoic acid, has the ability to alter methyl group metabolism by regulating important enzymes involved. High, pharmacological doses of all-*trans*-retinoic acid administered for 7-10 days have been shown to induce enzymes in methyl group metabolism. Currently there is no understanding of the dose-response relationship between this active vitamin A derivative and the enzymes it induces, nor the length of time required for induction in relationship to the mechanisms involved. Modulation of folate-dependent one-carbon and methyl group metabolism can aid in comprehension of the cycle and its regulation as well as the impact it has on the body in times of impaired health.

Thesis Organization

This thesis contains an introductory chapter that gives an overview of research on folate-dependent one-carbon and methyl group metabolism and how disruptions to various aspects of the pathway can ultimately affect health. The second chapter contains a paper submitted to and accepted by the *Journal of Nutrition* that illustrates the dose-response and time-course behavior of all-*trans*-retinoic acid on the aforementioned pathways. Chapter 3 gives closing thoughts and ideas for future research in this area. The appendices have supplemental data from the two studies in the second chapter and an additional study involving the impact of increased protein intake on folate-dependent one-carbon and methyl group metabolism.

Literature Review

Folate-dependent One-carbon and Methyl Group Metabolism

Folate-dependent one-carbon and methyl group metabolism are interrelated pathways that occur primarily in the liver and require a number of regulatory mechanisms to maintain function at an optimum level. This review describes the two pathways and their association with each other and will underscore how perturbations and disruptions may affect overall health.

Folate Metabolism

Folate's role in the body is to distribute carbon for DNA synthesis and donate methyl groups for methyl group metabolism. Folate is introduced into the body by foods containing the water-soluble vitamin found most often in dark, green leafy vegetables. Folic acid, a monoglutamyl and oxidized form of folate, is another dietary source that is found in fortified foods and supplements (1). The importance of folate in health has increased the commercial availability of folic acid-fortified products, which typically dominates a person's daily intake. The Recommended Dietary Allowance (RDA) of folate is 400 dietary folate equivalents (DFE)/day (1 DFE = 1 μg folate, 0.6 μg folic acid in fortified foods, or 0.5 μg in supplements) for men and women and between 150 and 300 DFE/day for children, depending on the age. Folate plays an influential role in pregnancy and its requirement is 600 DFE/day for pregnant women and 500 DFE/day for lactating women (2). Neural tube defects (NTDs) occur in the fetus and take place in 1 out of 1000 births in the United States and 1/3 of these are lost or terminated. There is a correlation between NTDs and folic acid but the mechanism of action is still unknown; however, it is strongly recommended for all women of childbearing age who are able to have children to consume 400 DFE/day (3). The malabsorption of folate has been associated with NTDs and may correlate to a higher rate of NTDs in some women (4).

Severe folate deficiency can result in megaloblastic anemia, a disorder characterized by the formation of large, nucleated cells erythrocyte precursors. The reduction in DNA synthesis prevents these cells from dividing properly to become mature red blood cells (5). Other disorders from folate deficiency that can occur are leukopenia (decreased number of white blood cells) and thrombocytosis (surplus of platelets) (6). DNA methylation may ultimately affect cancer in the body as Lucock *et al.* (7) points out that improved folate status may prevent the formation of cervical and colon cancer as well as bronchial malignancies. These are prime examples of the impact folate have on human health. Other detrimental effects can occur from a folate deficiency but these will be discussed in detail later in relationship to methyl group metabolism.

Folic acid is absorbed in the jejunum of the small intestine by hydrolysis with a conjugase intestinal enzyme located in the brush border (8). It is absorbed by both passive

diffusion and sodium-coupled carrier-mediated active transport before it is bound to folate-binding proteins that transform folic acid to tetrahydrofolate (THF) and transport the newly formed molecules in plasma to the liver where half of the folate pool is located; THF is converted primarily to 5-methyl-THF and 10-formyl-THF in the liver before being transported to peripheral tissues (8). THF can be converted to be able to donate carbons in the production of purines and pyrimidines (**Figure 1.1**). The addition of formate to THF via the enzyme 10-formyl-THF synthase creates 10-formyl-THF, which donates carbon twice during purine biosynthesis, providing the C-2 and C-8 in adenine and guanine as shown in **Figure 1.2**. A methyl group from serine transfers to THF via serine hydroxymethyltransferase with cofactor vitamin B₆ (in the form of PLP) and creates 5,10-methylene-THF and the by-product glycine. The activity of the enzyme thymidylate synthase donates the auxiliary methylene group from 5,10-methylene-THF to produce deoxythymidylate monophosphate, the precursor of thymine (6,9) (Figure 1.1, 1.2). Dihydrofolate (DHF) is produced from this reaction and is reduced back to THF via DHF reductase.

The folate derivative 5,10-methylene-THF can also be transformed by the enzyme 5,10-methylene-THF reductase (MTHFR) and the cofactor Vitamin B₂ (FADH₂) to 5-methyl-THF, a coenzyme used in the remethylation of homocysteine to produce methionine *de novo* by methyl group metabolism (**Figure 1.3**) (10). Control of MTHFR is inhibited allosterically by S-adenosylmethionine (SAM), an important metabolite within the methyl group metabolism (11). SAM helps control the influx of methyl groups from the folate-dependent one-carbon pool that are used for remethylation of homocysteine. If there is a sufficient amount of methionine to produce SAM, the increased SAM concentration inhibits the synthesis of 5-methyl-THF and the subsequent remethylation of homocysteine. There is more than one binding site for SAM on the MTHFR molecule and it will remain inactivated in the presence of SAM. The opposite action occurs if SAM levels decrease, producing an increase in MTHFR activity to generate 5-methyl-THF, which serves to remethylate homocysteine.

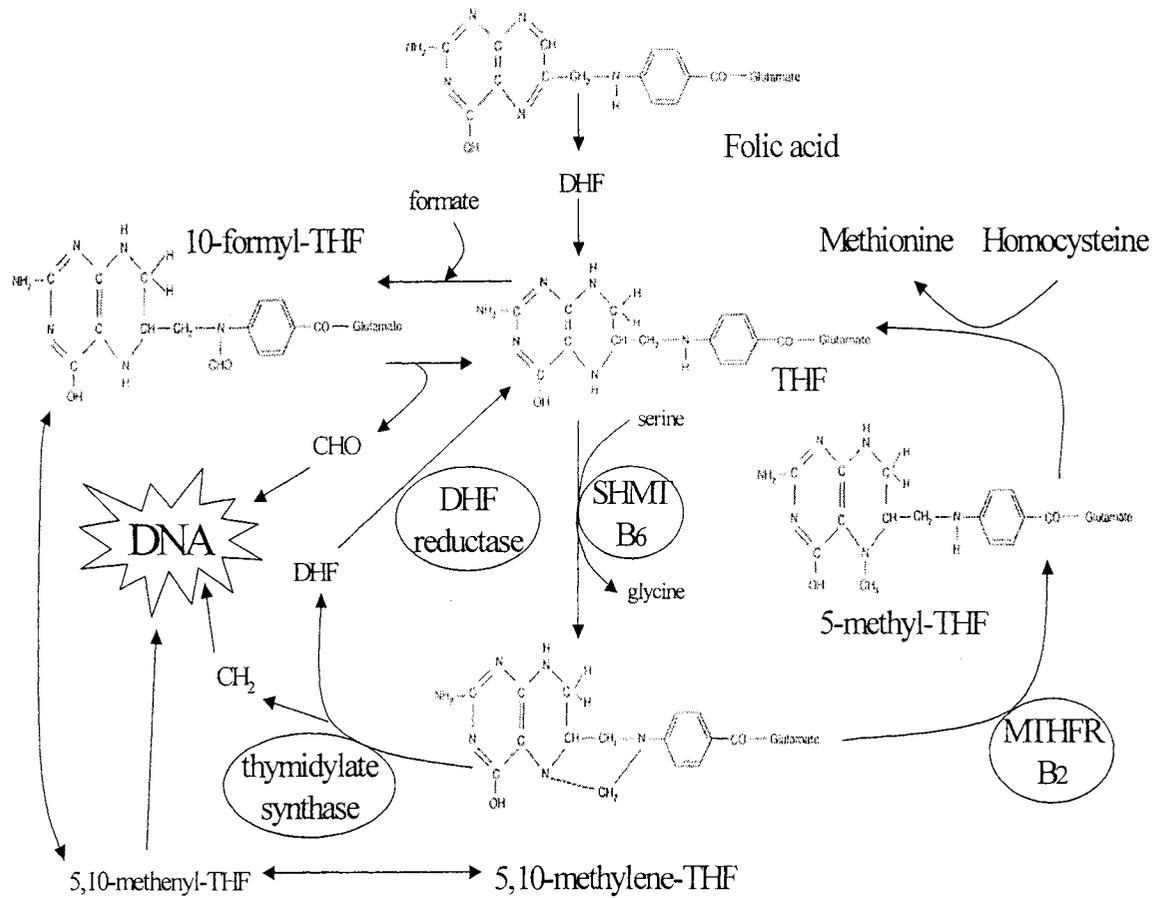
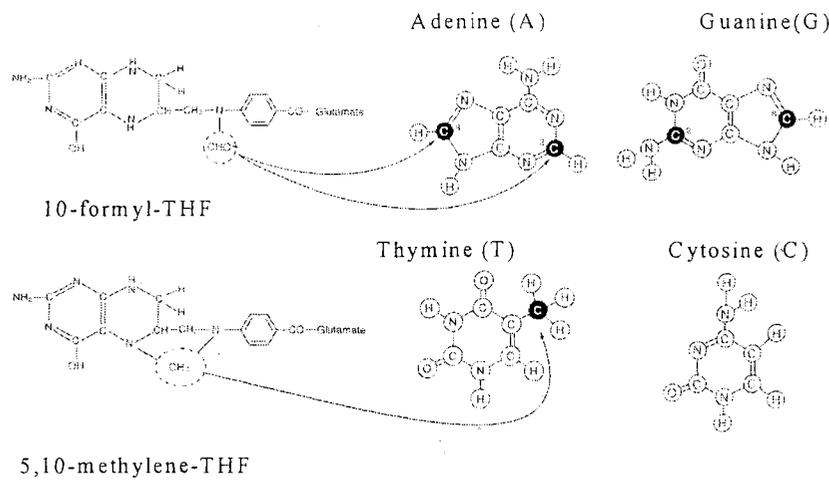


Figure 1.1 Diagram of the conversion of ingested folic acid into its active form, tetrahydrofolate (THF). THF can either be metabolized to 10-formyl-THF or 5,10-methylene-THF to donate carbons in the production of nucleic acids. 5,10-methylene-THF can also be metabolized via enzyme 5,10-methylene-THF-reductase (MTHFR) and cofactor B₂ to produce 5-methyl-THF, which remethylates homocysteine in methyl group metabolism. Abbreviations: DHF, dihydrofolate; MTHFR, 5,10-methylenetetrahydrofolate reductase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate.



Scott, J.M. & Weir, D.G. *J. of Cardio. Risk* 1998; 5:225.

Figure 1.2 Conversion of tetrahydrofolate (THF) yields either 10-formyl-THF or 5,10-methylene-THF. These two molecules donate carbons in the production of adenine, guanine, and thymine, the building blocks of DNA.

In the remethylation of homocysteine to achieve basal methionine concentration, the transfer of the methyl group from 5-methyl-THF to homocysteine produces THF and methionine via methionine synthase (MS), which requires the cofactor Vitamin B₁₂ (also known as cobalamin). The methyl group attaches to B₁₂ and becomes methylcobalamin, before being transferred to the homocysteine molecule (12). A deficiency of nutrients (i.e., B₁₂, folate) in combination with the irreversible action of MTHFR can cause the majority of folate to remain as 5-methyl-THF, creating a “methyl trap” that impairs folate-dependent reactions (13). A deficiency in B₁₂ will decrease MS activity by as much as 80% and aid in the “methyl trap” (13) along with unregulated MTHFR activity (14). Homocysteine can also be remethylated by betaine, a product of choline oxidation. This reaction is activated by the enzyme betaine:homocysteine methyltransferase (BHMT), an enzyme found only in the liver in all mammals while humans also possess BHMT in the kidney and lens (15). Choline, B₁₂, methionine, and folic acid are collectively known as lipotropes that are obtained in the diet (16). These nutritional compounds play an

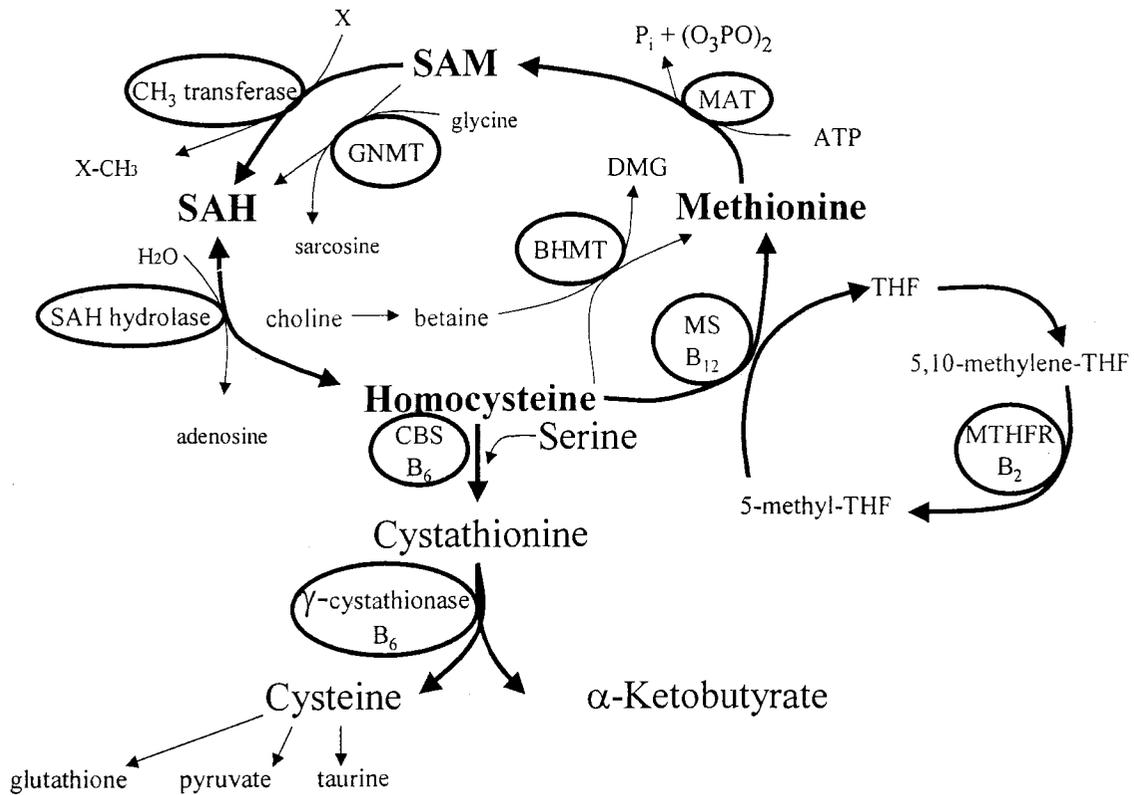


Figure 1.3 The enzyme methionine adenosyltransferase (MAT) activates S-adenosylmethionine (SAM), which methylates numerous biological compounds. Glycine N-methyltransferase (GNMT) is available to maintain the transmethylation potential. S-adenosylhomocysteine (SAH) is produced and hydrolyzed by SAH hydrolase to form homocysteine. Homocysteine can undergo transmethylation via the enzyme methionine synthase (MS) and coenzyme 5-methyl-THF or by the enzyme betaine:homocysteine methyltransferase (BHMT) and coenzyme betaine. If SAM concentrations are high, B₆-dependent cystathionine β-synthase (CBS) is activated to shunt homocysteine into the transsulfuration pathway and the eventual catabolism to cysteine and α-ketobutyrate. Abbreviations: BHMT, betaine:homocysteine methyltransferase; CBS, cystathionine β-synthase; DMG, dimethylglycine; GNMT, glycine N-methyltransferase; MAT, methionine adenosyltransferase; MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

important part in cellular metabolism and are essential for the synthesis and methylation of DNA, production of membranes, and metabolism of lipids. A deficiency of any of these compounds may lead to decreased immunocompetence, increased hepatic lipid accumulation, reduced xenobiotic metabolism, and influence the risk of promotion of cancer by upsetting folate-dependent one-carbon and methyl group metabolism (16).

The folate-dependent one-carbon cycle utilizes folate derivatives as important carbon-donating molecules in the synthesis of DNA as well as the production of methionine *de novo* in methyl group metabolism. The dietary intake of folate and folic acid is essential to maintain homeostasis within the cycle as coenzymes and enzymes react to optimize folate's role in the health process. Folate's relationship with methyl group metabolism can ultimately affect homocysteine metabolism and the importance of this relationship becomes apparent with its connection with cardiovascular disease.

Transfer of Methyl Groups into Methyl Group Metabolism

The relationship between folate and homocysteine has become a subject of intense research in recent years because of an association with cardiovascular disease. Homocysteine is a highly reactive amino acid and has been shown to promote oxidative stress by increasing hydrogen peroxide production in cell culture (17). Homocysteine is either remethylated by 5-methyl-THF or betaine or is further metabolized in the transsulfuration pathway by the irreversible enzyme cystathionine β -synthase (CBS) (15) (Figure 1.3). Homocysteine is protein-bound and present in low levels (7-24 $\mu\text{mol/L}$) in the blood and urine; consequently, disruptions to the folate-dependent and methyl group metabolism by congenital or nutritional disorders can cause an abnormal elevation of homocysteine concentration (as high as 200 $\mu\text{mol/L}$) in the plasma and urine. These states are known as hyperhomocysteinemia and hyperhomocysteinuria (18). The hepatocyte's homeostatic control in exporting accumulated homocysteine prevents cellular toxicity; however, the expelled homocysteine may leave vascular tissue in a threatening position because of its molecular characteristics (19). Homocysteine may jeopardize the vascular endothelium integrity by its ability to induce auto-oxidation of cholesterol and promote thrombosis (20). Kang *et al.* (21) showed that patients with coronary artery disease (CAD)

had significantly higher levels of homocysteine compared to those in the control group. The three enzymes involved with homocysteine metabolism work together and depend on the cellular concentration of SAM. MS or BHMT each metabolize approximately 27% of homocysteine, respectively, whereas the other 46% is transsulfurated by CBS (22). The regulatory control of SAM already demonstrated with MTHFR also inhibits BHMT from supplying methyl groups for methionine synthesis. An elevated SAM concentration also activates CBS to shunt homocysteine through the irreversible transsulfuration pathway (22,23).

Homocysteine has a vital role in the relationship between the folate-dependent one-carbon cycle and methyl group metabolism. It has been implicated in cardiovascular disease and the regulation of homocysteine metabolism depends on the cellular concentration of SAM and the enzymes involved with its metabolism. Homocysteine is either catabolized through the transsulfuration pathway or remethylated to form methionine and eventually form SAM, an important metabolite involved in the transmethylation of numerous biological compounds.

Methyl Group Metabolism

Methionine continues through the transmethylation pathway when ATP is hydrolyzed by the enzyme methionine adenosyltransferase (MAT) to release the adenosyl group that attaches to the sulfur of methionine, forming SAM (24). SAM can be seen as a function of methionine availability because of its biological activity and its ability to donate methyl groups in transmethylation reactions. It is found in both the cytosol and the mitochondria; however most of SAM-dependent transmethylation reactions take place in the cytosol (15).

SAM is a biologically important metabolite that donates methyl groups in over 100 reactions. Approximately 99% of SAM is demethylated in a multitude of reactions and 1% is decarboxylated for polyamine biosynthesis and eventually converted back to methionine. SAM is an active metabolite in that it helps to control the interactions between folate-dependent one-carbon cycle and methyl group metabolism. As previously stated, when SAM is in high concentration, it acts as an inhibitor to the enzyme BHMT to slow the production of methionine as well as inhibiting MTHFR activity to prevent the availability of 5-methyl-

THF for the remethylation of homocysteine. High SAM concentrations also facilitate the disposal of homocysteine through the transsulfuration pathway activating CBS and normalizing homocysteine concentration (25). As a universal donor of methyl groups, SAM donates its activated methyl group via a variety of methyltransferases and is converted to S-adenosylhomocysteine (SAH) (Figure 1.3). The ratio of SAM to SAH is indicative of the transmethylation potential due to the ability of SAH's potent inhibition of transmethylation reactions (26). A study by Caudill *et al.* (27) examined the SAM: SAH ratio and found there was a positive correlation between plasma homocysteine concentration and SAH levels in a CBS enzyme knockout mouse model. SAH may be a reliable index of transmethylation potential versus increased SAM concentration or the SAM: SAH ratio and that plasma homocysteine could be a good indicator of disturbance within methyl group metabolism. Products from the transmethylation reaction are too numerous to list, but the enzymes for these reactions are categorized into two groups: those involved in biosynthesis and degradation of bioactive amines, and those involved in bulk metabolic transformations (28). Major examples of the first group include epinephrine, phosphatidylcholine, and the modulation of DNA and RNA (29). An example of the second group involves creatine synthesis that constitutes the majority (~75%) of transmethylation reactions.

The products from the transmethylation reaction that occur in methyl group metabolism convey the importance of this pathway in the health process. Regulation is essential to maintain the SAM: SAH ratio to optimize the transmethylation potential; fortunately, there is an enzyme available to perform this function.

Properties of Glycine N-methyltransferase

The enzyme glycine *N*-methyltransferase (GNMT) uses the methyl group from SAM in the transmethylation reaction to produce sarcosine, a compound synthesized by that has no known metabolic function (Figure 1.3) (30). GNMT utilizes glycine as a methyl acceptor (31) and comprises about 0.9-3.0% of soluble protein in the liver (32). GNMT was first discovered in 1960 by Blumenstein and Williams and is known to be part of a system that regulates the SAM: SAH ratio (33). SAH is the product of SAM demethylation and has an instrumental role in regulation of the transmethylation reaction from its ability to inhibit

almost all of the methyltransferase enzymes. However, GNMT is not affected by SAH and optimizes the transmethylation potential (26). Heady and Kerr (34) found that the absence of GNMT activity in rabbit fetal liver and tumor tissue led to elevated levels of tRNA methyltransferase compared to normal adult tissue. The GNMT gene expression is also down-regulated in human hepatocellular carcinoma (HCC) cell lines as well as in liver tissue of HCC patients (35,36). GNMT has its own regulation, but in a different manner than other methyltransferases. Cook and Wagner (37) found that 5-methyl-THF can tightly bind GNMT but the function of this action was uncertain. Further research has shown that GNMT's highly specific binding to 5-methyl-THF serves as an allosteric inhibitor of GNMT activity and can occur in 5-methyl-THF concentrations that are similar to what is found in the liver (5.1×10^{-7} M showed virtually complete inhibition) (30). To summarize this regulation, low SAM levels result in increased MTHFR activity, thereby producing 5-methyl-THF that inhibits GNMT and allow SAM concentrations to increase and participate in biologically important transmethylation reactions (**Table 1.1**). When SAM concentrations increase beyond metabolic needs, it binds to MTHFR and inhibits its activity, thus decreasing the concentration of 5-methyl-THF. This action allows unbound GNMT to dispose of SAM and lower the SAM concentration.

Active GNMT is composed of four subunits with a denatured molecular weight of 31,500 kD, determined using the SDS-PAGE technique (31). Cell culture studies have shown that unbound GNMT monomers in the cytosol are covalently phosphorylated by a cAMP-dependent protein kinase and ATP, forming a tetramer and producing a ~2 fold increase in enzymatic activity (38). The disassociation of the tetrameric units produces a loss in activity and the monomer units can then be transported into the nucleus and bind to chromatin (39). Raha *et al.* (40) discovered that GNMT is also responsible for activity associated with the cytochrome P-4501A1 (CYP1A1) system within the nuclei of hepatoma H4IIE cell line. Formerly known as 4S polycyclic aromatic hydrocarbon (PAH)-binding protein, GNMT in dimeric form in the nucleus acts as a PAH-binding protein that has the ability to mediate the induction of CYP1A1 (41). GNMT plays an important role in methyl group metabolism and an uncontrolled increase in its activity can perturb folate-dependent one-carbon and methyl group metabolism as shown later in this thesis.

Table 1.1 Regulation and perturbations of enzymes in folate and methyl group metabolism

Enzyme	Influences on Activity
BHMT¹	Decrease: high SAM concentration; increased protein intake
CBS	Increase: high SAM concentration; glucocorticoids Decrease: B6 deficiency; insulin; genetic polymorphisms
GNMT	Increase: high SAM concentration; increased protein intake; retinoids; glucocorticoids; growth hormone Decrease: high 5-methyl-THF concentration
MS	Decrease: B12 deficiency; increased protein intake; genetic polymorphism
MTHFR	Decrease: high SAM concentration; B2 deficiency; genetic polymorphisms

¹Abbreviations: BHMT, betaine:homocysteine methyltransferase; CBS, cystathionine β -synthase; GNMT, glycine *N*-methyltransferase; MAT, methionine adenosyltransferase; MS, methionine synthase; MTHFR, 5,10-methylene-THF-reductase.

Homocysteine Metabolism and the Transsulfuration Pathway

During basal metabolic conditions, homocysteine cycles 1.5-2.0 times through the remethylation pathway (19). The enzyme SAH hydrolase catalyzes the hydrolysis of SAH to form homocysteine and adenosine (Figure 1.3). Because this enzyme is reversible and the equilibrium favors SAH synthesis, the rate of SAH hydrolysis depends on the removal of homocysteine either through remethylation or transsulfuration. As explained earlier, SAH is a potent inhibitor of methyltransferases and the SAM: SAH ratio is often used as an indicator of methylation capacity (26). As the ratio increases due to high SAM levels, CBS is activated to reduce homocysteine and normalize the SAM: SAH ratio (25). The complete transsulfuration pathway is not as wide spread in the body as folate-dependent one-carbon and methyl group metabolism and occurs only in the liver, kidneys, small intestine, and pancreas. When the B₆-dependent enzyme CBS is activated by a high SAM concentration,

the irreversible CBS enzyme attaches a serine to homocysteine to form cystathionine. CBS activity is also dependent on another factor; cell culture experiments have shown that heme produces an oxidative state, which increases CBS activity (42). The B₆-dependent enzyme γ -cystathionase cleaves cystathionine into 3 molecules: cysteine, α -ketobutyrate, and ammonium. Cysteine can be oxidized to taurine, used in the synthesis of glutathione, or as a precursor of pyruvate. Ammonium is excreted from the body through the kidneys and α -ketobutyrate is an intermediate substrate that is converted to propionyl-CoA and then to succinyl-CoA in the citric acid cycle.

Homocysteine is the branch point in folate-dependent one-carbon and methyl group metabolism and is either remethylated or undergoes transsulfuration in response to the cellular concentration of SAM. A few examples of the disruption of homocysteine metabolism have been demonstrated to show the relevance of homocysteine in response to health and disease. The regulation of these two interrelated pathways is important to address and should be considered in relation to diseases that may occur from disruptions.

Disruption of Folate and Methyl Group Metabolism

Under basal conditions, folate-dependent one-carbon and methyl group metabolism is tightly regulated and the body remains in a healthy state. When this system is perturbed, various diseases may occur; therefore, it is important to study the multitude of genetic and nutritional factors as well as pharmacological agents that can perturb its controlled environment. Folate and its role in methyl group metabolism is important in understanding how a disruption in the pathway can produce various detrimental outcomes. Table 1.1 summarizes some of the enzymes affected by disturbances in the pathway.

Genetic Enzyme Polymorphisms

Genetic enzyme polymorphisms are present in folate-dependent one-carbon and methyl group metabolism and these disruptions can cause health problems if left uncontrolled or perturbed by a nutritional deficiency. MS, CBS, and MTHFR enzyme polymorphisms have been extensively researched and different variants within each enzyme are established.

A common and extensively researched enzyme polymorphism that is found in folate metabolism is the enzyme MTHFR. This genetic polymorphism is caused by the C → T substitution at base pair 677 and causes a valine to substitute for an alanine, resulting in reduced stability under increases in temperature; therefore, thermolability is a fundamental characteristic of this polymorphism (43). This autosomal recessive mutation is characterized by hyperhomocysteinuria and hyperhomocysteinemia. Clinically, people with severe cases develop neurological abnormalities and atherosclerotic damage and often early death, while mild cases can be troubled with clinical vascular disease in adulthood (44). In a case study by Kanwar *et al.* (45), a mentally retarded 10-year-old child with homocystinuria exhibited no hepatic MTHFR activity. Additionally, the patient displayed distortions in the endothelial cells, deleterious changes in the brain and liver tissue, and demyelination of striated muscle and kidneys. A study investigating CAD cases showed that 6 out of 21 patients had thermolabile MTHFR that resulted in decreased enzyme activity. Of these 6 patients, 2 had high plasma homocysteine levels and suboptimal folate levels (44). A study by Silaste *et al.* (46) found that folate supplementation improved plasma homocysteine levels; healthy women (22-57 y) participated in a 5-week crossover study in which they consumed high (600 µg/day) and low (220 µg/day) folate diets. These women were tested for polymorphic enzymes and those with the MTHFR mutation on the high folate diet showed a decrease in plasma homocysteine levels but a continued suboptimal plasma folate level. The high folate diet did not affect plasma homocysteine levels of people with CBS or MS polymorphisms. A study performed by Kluijtmans *et al.* (47) also examined the three enzyme polymorphisms and their different variants in 452 young adults. The frequency of the enzyme polymorphisms MTHFR (2 types 677TT and 1298CC) occurred 13.5% and 10.6% respectively, MS (2756GG and 66GG) occurred 2.0% and 29.6% respectively, and CBS (1 type 844ins68 WI) occurred 17.7%. Of these enzyme deficiencies, MTHFR 677TT was the only one to have an increase in subjects' plasma homocysteine concentration and a decrease in serum folate levels. The authors conclude that 35% of the variability involved in plasma homocysteine concentration could be attributed to folate and B₁₂ concentrations, implicating the importance of nutrition on human health, even at a young age.

The MS enzyme polymorphism has been researched for its impact on the remethylation of homocysteine in methyl group metabolism but has not been shown to have the detrimental impact that MTHFR and CBS polymorphisms display (48,49). However, the genetic polymorphism of the CBS enzyme does perturb homocysteine metabolism and can be detrimental. The CBS enzyme deficiency is caused by an autosomal recessive mutation and is the most common cause of hyperhomocysteinemia with fasting plasma homocysteine levels as high as 200 $\mu\text{mol/L}$. These elevated levels cause mental retardation, Marfan-like habits, osteoporosis, ectopic lentis, and thromboembolic disease. A CBS knockout mouse model using a homozygous mutant exerted a dramatic increase in plasma homocysteine levels and increased SAH concentration in various tissues compared to heterozygous wild-type mice (50). A clinical report by Yaghmai *et al.* (51) described the symptoms of a 10-year-old patient with CBS deficiency that was on an unrestricted diet and was undergoing betaine therapy to treat hyperhomocysteinemia. Methionine toxicity developed and she suffered from cerebral edema, pancreatitis, and mild bradycardia. After stopping the betaine therapy, restricting methionine intake and supplementing her diet with folate, B₆, and B₁₂, her plasma methionine and homocysteine levels returned to baseline and all adverse symptoms disappeared.

The conversion of homocysteine to cystathionine occurs in the brain and impaired CBS activity and folate deficiency in brain cells may cause accumulated homocysteine to promote excitotoxicity, stimulating N-methyl-D-aspartate receptors and damaging neuronal DNA. Such conditions may disrupt brain development in children and could have a role in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, schizophrenia, and depression (52).

There are genetic enzyme polymorphisms present throughout the one-carbon cycle and methyl group metabolism. The lack of activity in the enzymes CBS and MTHFR has been implicated in increased homocysteine concentrations in the plasma and urine, which are correlated to a heightened risk for cardiovascular disease. Researching the influence of diet on genetic enzyme polymorphisms is ongoing and is seen as an important aspect in preventing disease.

Nutritional Deficiencies

If homocysteine metabolism is disrupted by a genetic disorder, SAH concentration increases, perturbing the SAM: SAH ratio and disrupting the cellular methylation potential (26). A deficiency in folate, methionine, choline, or B₁₂ could exacerbate this situation in the remethylation pathway of homocysteine while a deficiency in B₆, which aids in the activity of CBS and γ -cystathionase could decrease the shuttling of homocysteine through the transsulfuration pathway (53).

Hyperhomocysteinemia has been identified as an independent risk factor for vascular disease and multiple studies have looked at folate intake and its influence on plasma homocysteine concentration. A study by Pancharuniti *et al.* (54) compared 108 white males with angiographically demonstrated CAD to age-related controls and concluded that an increased risk of CAD was present in patients with the lowest level of plasma folate concentration, which was inversely related to their plasma homocysteine concentration. In addition, vitamin B₁₂ plasma levels had no correlation with CAD. Van Oort *et al.* (55) demonstrated that folate supplementation in 316 healthy men and women (50-75 y) decreased plasma homocysteine levels in a dose-dependent fashion with 392 $\mu\text{g}/\text{day}$ as the lowest dose to produce a positive homocysteine reduction. Brouwer *et al.* (56) examined the impact of natural folate versus folic acid supplements in healthy men and women (18-45 y). The subjects received a dietary intake of either a placebo and low folate diet ($\sim 210 \mu\text{g}/\text{day}$), a placebo and high folate diet mainly from vegetables and citrus fruit ($\sim 560 \mu\text{g}/\text{day}$), or a folic acid supplement and low folate diet ($\sim 560 \mu\text{g}/\text{day}$). After 4 weeks, showed that plasma folate status improved and plasma homocysteine concentrations decreased in the high folate/folic acid groups compared to the control group. These results indicate that dietary folate and folic acid supplements are both able to reduce plasma homocysteine levels.

The lack of dietary methyl groups has been linked to tumor induction and is well documented. The lipotropes methionine, choline, and folate are essential to the folate-dependent one carbon pool and methyl group metabolism and when a diet is deficient in these nutrients, it will induce tumors without the aid of an outside carcinogen (57). A study by Miller *et al.* (58) examined the folate-repletion activity in rats that were fed a folate-deficient diet for 4 weeks. There was a 10-fold increase in plasma homocysteine

concentration and a 3-fold decrease in hepatic SAM levels in folate-deficient rats compared to the control group. The authors attribute these findings to an impaired remethylation capacity and inactivation of CBS activity. Interestingly, folate repletion returned the homocysteine levels to baseline correlating to the incremental folate doses given. Additionally, ethionine injections were administered to the folate-depleted rats for 3 days to test the efficacy of inducing homocysteine catabolism without increasing SAM. Ethionine was converted to S-adenosylethionine and caused a 300% increase in CBS activity and lowered the plasma homocysteine levels.

Cook *et al.* (59) demonstrated the impact of rats fed a methyl-deficient diet in which homocysteine was added in place of methionine in the diet. Weights significantly decreased throughout the 5 weeks in rats on the deficient diet even though both groups consumed similar amounts of diet. The livers of the methyl-deficient group had an increased fat concentration, decreased GNMT activity, slightly decreased SAM concentration, and SAH concentration was elevated as homocysteine was rapidly converted to SAH. The treatment group ingested the control diet for the last week and showed an increase in GNMT activity. The perturbations seen in methyl group metabolism from a methionine-deficient diet has implications on DNA that affect the entire health process. The disruption of SAM's ability to methylate DNA will cause progressive dysregulation of DNA methylation, priming the stage for tumor promotion (60).

A deficiency in the dietary intake of the folate can have a profound effect on the folate-dependent one-carbon pool and methyl group metabolism. The preceding paragraphs examined the importance of dietary folate or folic acid in the regulation of homocysteine metabolism and its inverse relationship to plasma homocysteine concentration. Nutritional deficiencies of the lipotropes methionine and choline in addition to low folate intake can disrupt enzyme activity and the cellular concentration of metabolites within the interrelated cycles and are implicated in hepatocarcinoma. When additional perturbations are present, the risk of disease is heightened. Whether the disruption comes from an exogenous source (i.e., drugs, alcohol) or comes from endogenous conditions (i.e., hormones), the harmful fluctuations to folate and methyl group metabolism can further exacerbate the already taxed pathway.

Hormonal Modulation

Fluctuations in hormones influence enzyme activity and can perturb coenzymes within methyl group metabolism. Glucocorticoids are hormones secreted from the adrenal cortex that elevate blood glucose and can mediate a diabetic-like state. The induction of diabetes or the application of glucocorticoids may have an effect on CBS activity and its protein levels. Cell culture research using the H4IIE cell line showed that synthetic glucocorticoids increased CBS enzyme protein and mRNA levels while insulin inhibited this effect, providing insight that insulin may have an influential role in homocysteine metabolism (61). Ratnam *et al.* (61) used a streptozotocin-induced diabetic rat model that exhibited increased hepatic CBS activity and mRNA protein that was reversed by the application of insulin; the induction of CBS activity and mRNA was not found in the kidneys. Hyperglucagonemic rats (which displayed increased CBS activity and protein abundance) exhibited no change in hepatic methionine concentration but there was a significant decrease in both plasma and hepatic homocysteine levels (62). The diabetic drug troglitazone, which enhances insulin activity, was administered to lean and overweight rats and the drug may have aided in the hepatic uptake of homocysteine from the plasma, decreasing plasma homocysteine concentrations and causing a dramatic increase in hepatic CBS enzyme activity in both rat groups (63). However, these homocysteine reductions may not occur in diabetic cases where renal insufficiency is present, a condition strongly associated with hyperhomocysteinemia (64).

While the enzyme GNMT is found predominately in the periportal region of the liver, its prevalence in methyl group regulation is also found in the proximal convoluted tubules of the kidney and the exocrine pancreas of both rabbit and rat, regions that are known for gluconeogenic activity (65,66). In alloxan-induced diabetic sheep, the activity of GNMT was significantly increased as well as γ -cystathionase compared to the control, suggesting an exaggerated homocysteine catabolism in a diabetic state (67). Jacobs *et al.* (62) showed similar conclusions using hyperglucagonemic rats that resulted in increased hepatic GNMT, CBS, and γ -cystathionase enzyme activity, concluding that glucagon increases intracellular concentrations of cAMP and was responsible for the increased CBS mRNA levels. Rowling and Schalinske (68) showed in cell culture that the treatment of the H4IIE cell line with

cAMP appeared to attenuate glucocorticoid-mediated induction of GNMT protein abundance. In the rat model, the synthetic glucocorticoid dexamethasone induced GNMT activity and protein abundance as it decreased plasma homocysteine levels versus the control treatment. Aida *et al.* (69) found that the administration of growth hormone on hypophysectomized male mice reduced GNMT mRNA expression whereas female mice were not affected as much, indicating a difference between male and female mice while growth hormone is seen as another influence on GNMT regulation.

The regulation of enzymes in methyl group metabolism ultimately affects the cellular concentration of metabolites that are involved in the creation of biological products. Whether it involves GNMT activity in regards to SAM in the transmethylation reaction or homocysteine metabolism in relation to CBS and -cystathionase activity, the influence of hormones to these enzymes in a derogatory manner can perturb the pathway and may be linked to cardiovascular disease and cancer. Disruptions to the folate-dependent one-carbon pool and methyl group metabolism from the addition of exogenous factors can also be implicated in the development of disease.

Drug-Nutrient Interactions

The classic folate antagonist drug model is found in the area of cancer treatment. Folate analogs are often given to slow the increased rate of DNA synthesis in cancerous cells. An example of this is methotrexate, an anticancer drug that inhibits the conversion of DHF to THF. The use of methotrexate results in an intracellular depletion of folate metabolites and reduced the carbon flow from 5-methyl-THF to homocysteine (70).

The application of antiepileptic drugs has frequently exhibited low plasma folate and high plasma homocysteine concentrations. Apeland *et al.* (71) found a decrease in plasma vitamin B₆ concentration in patients taking antiepileptic drugs; implying abnormal homocysteine metabolism and increasing the chance of diseases associated with high homocysteine concentrations. Pregnant women taking antiepileptic drugs have an increased chance of NTDs and Weber *et al.* (72) has shown the importance of folic acid supplementation as an effective preventative measure to this population.

Another factor that results in perturbations to methyl group metabolism is ethanol intake. In long-term administration, it has been shown that as the catabolism of methionine is increased by decarboxylation, leading to decreased SAM levels, and increased BHMT activity concurrent with decreased MS activity. GNMT activity decreased but only in the 8th week of ethanol intake versus weeks 2 and 4 (73). After a single dose of ethanol (3 g/kg BW), hepatic SAM concentration decreased in rats concurrently with inhibited CBS activity; enzymes involved in glutathione production were inhibited and decreased hepatic glutathione concentration did not return to control levels for 24 hr (74).

The influence of drugs and alcohol on folate and methyl group metabolism is profound. The overconsumption of alcohol is well known for its effect on the liver and antiepileptic drugs decrease plasma folate levels, subsequently leading to increased plasma homocysteine levels and the increased risk for NTDs. There are other drugs that are prescribed by doctors that have an impact on methyl group metabolism and the folate pool that are not as well known. Retinoids used in cancer treatment and dermatology are emerging as one of these such drugs.

Retinoid Implications

Retinoids are derived from the fat-soluble vitamin A. Vitamin A is important in growth and overall health but is considered toxic in high doses. A study by Fell and Steele (75) found that the intake of 1000 IU (0.3 mg) of retinol acetate/gm diet for 10-14 days suppressed MTHFR activity, resulting in decreased 5-methyl-THF levels and decreased SAM concentrations. The vitamin A derivative 13-*cis*-retinoic acid (CRA), which is more biologically active than retinol acetate, can increase serum triglyceride levels and hepatic lipids while perturbing methyl group metabolism by reducing hepatic SAM concentration and affecting the SAM: SAH ratio (76). However, CRA's increased biological activity has made it an effective agent in the dermatology field. CRA's success in the treatment of common skin disorders such as severe acne and psoriasis has produced synthetic retinoids like Accutane® and Tegison® and its use is widespread in dermatology. The frequent causes of acne are reduced from retinoid facilitation on the de-differentiation of sebaceous glands, sebum gland production suppression, and promotion of shedding keratinocytes (77). These

drugs are taken orally and are given when topical treatments do not work. The number of retinoid prescriptions has steadily increased in the past ten years as doctors are prescribing therapeutic retinoids for moderate acne as well (78). Oral retinoids are administered in doses ranging from 0.5-2.0 mg/kg BW (2-7 $\mu\text{mol/kg BW}$) for an average of 4-5 months. Adverse effects are seen in 100% of users in the form of skin and lip dryness, eczema, and hair shedding which subside within 3 weeks of treatment. Plasma triglyceride levels as well as cholesterol has been shown to increase with synthetic retinoid use, therefore patients are screened before starting retinoid therapy and at 2-4 week intervals (77). The most biologically active retinoid is all-*trans*-retinoic acid (ATRA) and is used clinically in the treatment of acute promyelocytic leukemia in a dosing regime similar to CRA. Rowling and Schalinske (79) found an increase in GNMT activity and protein abundance after 10 days of ATRA treatment (30 $\mu\text{mol/kg BW}$) in rats. Retinoids in the form of retinyl palmitate, CRA, and ATRA have all been shown to induce both GNMT activity and protein abundance and caused DNA hypomethylation in rats when administered in pharmacological doses for a short time period (10 d) (80). Even though the folate-dependent one-carbon pool and methyl group metabolism is found in the pancreas and kidneys, McMullen *et al.* (81) did not show increases in GNMT activity or protein abundance in rats in these two tissues as seen in the liver after ATRA application (30 $\mu\text{mol/kg BW}$ for 10 d). There was also an increase in GNMT activity in male versus female rats even though both groups were dosed and treated in the same manner.

Researching the impact that retinoids have on methyl group metabolism is in its initial stages and the overall outcome remains to be seen. A relationship between the pharmacological dose of ATRA on rats and the doses given in clinical settings has not been established. The studies in Chapter 2 include a dose-response and a time-course study of ATRA's affect on folate and methyl group metabolism in an attempt to bridge this gap. The folate-dependent one-carbon pool and methyl group metabolism's involvement in NTDs, cancer, and cardiovascular disease is prevalent but the mechanisms in the progression of these diseases are not completely known. Recently, research has shown that hormones involved in glucose metabolism have an effect on methyl group metabolism and this relationship may help explain anomalies within both pathways. Disruption to methyl group

metabolism by ATRA has shown to be an effective model in understanding this pathway and may someday provide insight to the diseases and disorders associated with the folate-dependent one carbon pool and methyl group metabolism.

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CHAPTER 2. ALL-*TRANS*-RETINOIC ACID RAPIDLY INDUCES GLYCINE *N*-METHYLTRANSFERASE IN A DOSE-DEPENDENT MANNER AND REDUCES CIRCULATING METHIONINE AND HOMOCYSTEINE LEVELS IN RATS

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Marlies K. Ozias and Kevin L. Schalinske

Abstract

Glycine *N*-methyltransferase (GNMT) regulates the methyl group supply for *S*-adenosylmethionine-dependent transmethylation reactions. Retinoids have been shown to perturb methyl group metabolism by increasing the abundance and activity of GNMT, thereby leading to the loss of methyl groups. Previous studies used pharmacological doses (30 $\mu\text{mol/kg}$ BW) of various retinoids administered daily for a total of 10 d. Here, we examined the dose- and time-dependent relationship between all-*trans*-retinoic acid (ATRA) administration and induction of GNMT, as well as determining additional indices of methyl group and folate metabolism. For the dose-response study, rats were given either 0, 1, 5, 10, 15 or 30 μmol ATRA/kg BW for 10 d. For the time-course study, rats received 30 μmol ATRA/kg BW for 0, 1, 2, 4, or 8 d. A significant increase (105%) in GNMT activity was observed with doses as low as 5 $\mu\text{mol/kg}$ BW, whereas maximal induction (231%) of GNMT activity was achieved at 30 $\mu\text{mol/kg}$ BW. Induction of hepatic GNMT by ATRA was rapid, exhibiting a 31% increase following a single dose (1 d) and achieving maximal induction (95%) after 4 d. Plasma methionine and homocysteine concentrations were decreased 42 and 53%, respectively, in ATRA-treated rats compared to control values. In support of this finding, the hepatic activity of methionine synthase, the folate-dependent enzyme required for homocysteine remethylation, was elevated 40% in ATRA-treated rats. This work demonstrates that ATRA administration exerts a rapid effect on hepatic methyl group, folate, and homocysteine metabolism at doses that are within the therapeutic range used by humans.

Introduction

Methyl group and folate-dependent one-carbon metabolism play an important role in the methylation of phospholipids, neurotransmitters, proteins, and nucleic acids (1,2). These interrelated pathways provide the needed methyl groups for numerous transmethylation reactions that ultimately affect human health. Transmethylation reactions in methyl group metabolism results in the production of S-adenosylhomocysteine (SAH) from S-adenosylmethionine (SAM) (**Figure 2.1**). Following hydrolysis of SAH, the resulting homocysteine can undergo transsulfuration to produce cystathionine, which can eventually be metabolized to cysteine and glutathione, as well as other important compounds. Alternatively, remethylation of homocysteine and the concomitant conversion of 5-methyltetrahydrofolate (5-methyl-THF) to tetrahydrofolate (THF) from the folate-dependent one-carbon pool results in the regeneration of methionine, the precursor for SAM (2). An inability to metabolize homocysteine by either transsulfuration and/or transmethylation leads to increased plasma levels, a characteristic associated with thromboembolic diseases and disruption of vascular wall maintenance (3,4).

A number of regulatory mechanisms function to maintain optimal metabolism of folate, methyl groups, and homocysteine. SAM allosterically inhibits 5,10-methylenetetrahydrofolate reductase (MTHFR), the enzyme that irreversibly produces 5-methyl-THF for the subsequent remethylation of homocysteine, a reaction that requires the B₁₂-dependent enzyme methionine synthase (MS) (5,6). SAM also serves as a positive modulator of cystathionine β -synthase, the enzyme that catalyzes the formation of cystathionine from homocysteine as the initial step in the transsulfuration pathway (7). Regulation is also maintained by the action of glycine *N*-methyltransferase (GNMT), a key cytosolic enzyme that converts glycine to sarcosine to regulate the utilization of methyl groups and optimize the ratio of SAM: SAH (2,8). Because SAH is a potent inhibitor of most methyltransferases (9), the ratio of SAM: SAH and its regulation by GNMT controls transmethylation processes. GNMT is also a folate-binding protein that is allosterically inhibited by the folate coenzyme 5-methyl-THF (10,11). In addition to allosteric regulation,

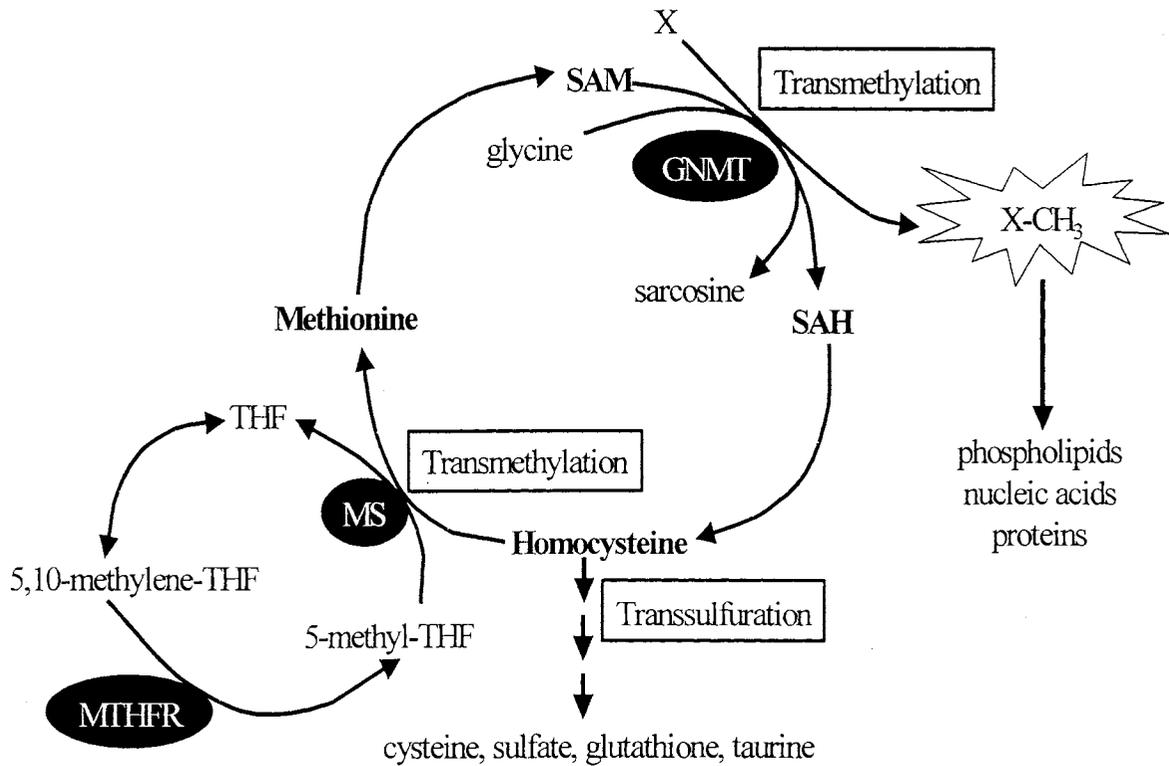


FIGURE 2.1 Methyl group, homocysteine and folate metabolism. S-adenosylmethionine (SAM) serves as the methyl group donor in numerous transmethylation reactions ($X \rightarrow X-CH_3$), resulting in the formation of S-adenosylhomocysteine (SAH) and eventually be converted to homocysteine. Homocysteine can be catabolized through the transsulfuration pathway, or remethylated back to methionine by the action of the B₁₂-dependent enzyme, methionine synthase (MS). This latter reaction is dependent on the one-carbon pool to supply the necessary methyl group from 5-methyl-THF, irreversibly generated by the reduction of 5,10-methylene-THF via 5,10-methylene-THF-reductase (MTHFR). Glycine N-methyltransferase (GNMT) regulates the SAM:SAH ratio and transmethylation potential by disposing of methyl groups in the form of sarcosine under conditions of excess. For further regulation of methyl group supply, SAM is an allosteric inhibitor of MTHFR, whereas 5-methyl-THF inhibits the activity of GNMT. Abbreviations: GNMT, glycine N-methyltransferase; MS, methionine synthase; MTHFR, 5,10-methylene-THF-reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

Thus, modulation of any of these regulatory mechanisms has the potential to disrupt the metabolism of folate, methyl groups, or homocysteine.

We have shown that retinoids such as all-*trans*-retinoic acid (ATRA) have the ability to perturb methyl group metabolism by increasing the activity and abundance of GNMT, thereby leading to the loss of methyl groups required for other biological processes (13-16). These studies utilized large doses of ATRA (30 $\mu\text{mol/kg}$ BW) administered for at least 7-10 d. Thus, it is important to examine lower doses that are more relevant to human usage, as well as the length of treatment with ATRA. In the work presented here, we examined the dose- and time-dependent relationship between ATRA and the induction of GNMT as well as parameters associated with folate and homocysteine metabolism.

Materials and Methods

Chemicals and reagents.

Reagents were obtained from the following: *S*-adenosyl-*L*-[methyl- ^3H]methionine, New England Nuclear (Boston, MA); phenylmethylsulfonyl fluoride and ATRA, Calbiochem (La Jolla, CA); goat anti-rabbit immunoglobulin G horseradish peroxidase, Southern Biotechnology (Birmingham, AL); ECL Western blotting detection reagents, Amersham Pharmacia (Piscataway, NJ); and *S*-adenosyl-*L*-methionine, Sigma Chemical (St. Louis, MO). GNMT antibody was provided by Y-M.A. Chen, National Yang-Ming University, Taipei, Taiwan (17). All other chemicals were of analytical grade.

Animals and diet.

All animal experiments were approved by and conducted in accordance with Iowa State University Laboratory Animal Resources Guidelines. For all studies, male Sprague-Dawley (Harlan Sprague-Dawley, Indianapolis, IN) rats (50-74 g) were housed in plastic cages in a room with a 12-h light: dark cycle and fed a control diet as described (16). Rats were acclimated to the control diet and the oral administration of corn oil for 3 d. For the dose-response study, ATRA was orally administered at 0, 1, 5, 10, 15, and 30 $\mu\text{mol/kg}$ BW using corn oil as the vehicle for a total of 10 d. For the time-course study, rats received

either ATRA (30 $\mu\text{mol/kg}$ BW) for 1, 2, 4, or 8 d or vehicle (0 and 8 d). At the end of the treatment period, rats were anesthetized and whole blood was obtained via cardiac puncture for determination of plasma methionine and homocysteine concentrations. Liver samples were obtained for determining the enzymatic activity and abundance of GNMT and folate coenzyme concentrations, as well as the enzymatic activity of MTHFR and MS.

Measurement of GNMT activity.

GNMT enzymatic activity was assayed as described by Cook and Wagner (10) with minor modifications. Portions of liver were homogenized in three volumes of ice-cold phosphate buffered (10 mmol/L, pH 7.0) sucrose (0.25 mol/L) containing 1 mmol/L EDTA, 1 mmol/L sodium azide, and 0.1 mmol/L phenylmethylsulfonylfluoride. After centrifugation (20,000 \times g for 30 min), supernatants were stored with 2-mercaptoethanol (10 mmol/L) at -70°C . The assay contained 1 mol/L Tris buffer (pH 9.0), 5 mmol/L dithiothreitol, 10 mmol/L glycine, and 1 mmol/L *S*-adenosyl-*L*-[methyl- ^3H]methionine (47.7 kBq/ μmol) and the reaction was initiated with 250 μg of the protein supernatant. For the determination of total soluble protein in the tissue extract, a commercial kit (Coomassie Plus, Pierce, Rockford, IL) based on the method of Bradford (18) was used with bovine serum albumin as a standard.

Measurement of GNMT protein abundance.

GNMT protein abundance was determined using immunoblotting procedures previously described (16). A 10-20% gradient SDS-polyacrylamide gel was used for the separation and subsequent determination of the GNMT monomer subunit (32 kDa) abundance. After separation, proteins were transferred to a nitrocellulose membrane and incubated for a minimum of 6-24 h with a monoclonal GNMT antibody (17) at 4°C followed by 1 h incubation at room temperature with goat anti-rabbit horseradish peroxidase secondary antibody. Chemiluminescence detection was used to determine GNMT protein abundance following exposure to Kodak X-Omat AR film. Densitometric analysis was performed using SigmaGel Software (SPSS, Chicago, IL).

Homocysteine analysis.

Plasma total homocysteine concentrations were determined according to the method of Ubbink *et al.* (19). In brief, heparinized blood from the cardiac puncture was immediately centrifuged ($3,800 \times g$ for 6 min) and plasma samples were stored at -70°C until derivatization. For derivatization, N-acetylcysteine (1 mmol/L) was added as an internal standard to 300 μL of plasma. An equal volume of 100 mL/L tributylphosphine in dimethylformamide was added followed by incubation at 4°C for 30 min. The reaction was stopped with 100 mL/L trichloroacetic acid – 1.0 mmol/L EDTA and following centrifugation at $1,000 \times g$ for 5 min, the supernatant was added to a solution containing borate buffer (0.125 mol/L, pH 9.5), sodium hydroxide (1.55 mol/L), and 4-fluoro-7-sulfobenzofurazan (1 g/L). Samples were filtered and analyzed by HPLC using fluorometric detection and a mobile phase consisting of 960 mL/L potassium phosphate (0.1 mol/L, pH 2.1) buffer and 40 mL/L acetonitrile.

Determination of hepatic folate coenzyme concentrations.

THF and 5-methyl-THF were determined using HPLC and fluorometric detection according to Rebello (20) with some minor modifications. Briefly, portions of liver were homogenized in 4 volumes of ice-cold sodium acetate buffer (0.1 mol/L, pH 4.9) containing 5 mL/L ascorbate and 20 mmol/L 2-mercaptoethanol under a steady stream of nitrogen, tightly capped, and stored at -70°C until analysis. Samples were placed in a boiling water bath for 60 min and following centrifugation at $20,000 \times g$ for 10 min, rat serum conjugase was added to an aliquot of the resulting supernatant and incubated in a shaking water bath for 1 h at 37°C . Following activation of Sep-Pak NH_2 columns with acetonitrile and sodium acetate buffer (16 mmol/L, pH 4.5), samples were applied and washed with acetate buffer and sodium phosphate (0.1 mol/L) containing 50 mmol/L 2-mercaptoethanol. Folate coenzymes were separated on a Phenyl Radial-Pak column (Waters, Milford, MA) and quantified using fluorometric detection. A gradient mobile phase operated at 2.0 mL/min consisted of: 760 mL/L sodium phosphate (0.1 mol/L, pH 7.5) – 240 mL/L acetonitrile for 4 min; a linear gradient (2 min) to 500 mL/L – 500 mL/L and maintained from 6 to 10 min; a linear gradient (2 min) to 100 mL/L – 900 mL/L and maintained from 12-16 min; and a

linear gradient (2 min) back to initial conditions (760 mL – 240 mL/L) for up to 20 min to re-equilibrate the column.

MTHFR determination.

The activity of MTHFR was determined in liver samples as previously described (21,22). Briefly, portions of liver were homogenized in 4 volumes of ice-cold potassium phosphate buffer (0.5 mol/L, pH 7.2) containing 0.1 mmol/L dithiothreitol, centrifuged at $40,000 \times g$ for 30 min, and stored at $-70\text{ }^{\circ}\text{C}$ until analysis. The assay mixture contained 1.0 mol/L potassium phosphate (pH 6.7), 0.5 mol/L sodium ascorbate, 0.1 mol/L EDTA, 10 mmol/L menadione, 1.0 mmol/L FAD, and 25 mmol/L 5- ^{14}C]-THF (74 Bq/ μmol). The reaction was initiated with the protein supernatant and incubated for 30 min at $30\text{ }^{\circ}\text{C}$. The reaction was terminated with dimedone (3 g/L in 1 mol/L sodium acetate) and samples were placed in a boiling water bath for 5 min. After being placed on ice, a 1.0 mL aliquot was extracted with toluene and a 1.0 mL aliquot of the resulting supernatant ($500 \times g$ for 5 min) was subjected to liquid scintillation counting.

MS determination.

The activity of MS was determined as previously described (23). Briefly, liver supernatant samples were incubated at $37\text{ }^{\circ}\text{C}$ for 1 h in a reaction mixture containing sodium phosphate buffer (500 mmol/L, pH 7.5), cyanocobalamin (1.3 $\mu\text{mol/L}$), dithiothreitol (1 mol/L), SAM (10 mmol/L), 2-mercaptoethanol (82.4 mmol/L), homocysteine (100 mmol/L), and 15 mmol/L 5- ^{14}C]-THF (6.44 kBq/ μmol). Reactions were stopped with the addition of ice-cold water and samples were immediately applied to AG 1-X8 (Cl form) resin columns (Bio-Rad, Hercules, CA). Flow through fractions (3 mL total) were collected and subjected to liquid scintillation counting.

Amino acid analysis.

Amino acids from plasma samples were prepared by using a EZ:faast™ GC-MS analysis kit (Phenomenex, Torrance, CA). Derivatized samples were analyzed by gas chromatography-mass spectrometry (Agilent Technologies, 6890/5973).

Statistical analysis.

The mean values of each treatment group were subjected to a one-way ANOVA (24). If the ANOVA was significant ($P < 0.05$), the means were compared using Fisher's least significant difference procedure.

Results

ATRA induced hepatic GNMT in a dose-dependent manner.

Hepatic GNMT activity (**Figure 2.2A**) and abundance (**Figure 2.2B**) increased in response to graded levels of ATRA. For the 10-d treatment period, 5 $\mu\text{mol/kg BW}$ was the lowest dose of ATRA that significantly increased (105%) GNMT activity. GNMT activity continued to increase with respect to the dose of ATRA administered, achieving a maximal response (231%) at 30 $\mu\text{mol/kg BW}$. Immunoblotting analysis indicated that the changes in activity were also reflected in GNMT protein abundance.

Hepatic GNMT was rapidly induced by administration of ATRA.

GNMT activity was significantly increased 31 and 62% following a single dose of ATRA (30 $\mu\text{mol/kg BW}$) compared to either group of untreated rats (d 0 and 8, respectively) (**Figure 2.3A**). Maximal induction was achieved on d 4 and 8, exhibiting a 95 and 83% increase, respectively, compared to control values on d 0. Hepatic GNMT protein abundance also increased as a function of treatment time with ATRA (**Figure 2.3B**).

Plasma homocysteine and methionine concentrations were significantly decreased by ATRA treatment.

ATRA treatment resulted in a 53% decrease in plasma homocysteine levels (**Figure 2.4**). Similarly, plasma methionine concentrations were decreased 42% from 50.3 to 29.1 $\mu\text{mol/L}$ in control and ATRA-treated rats, respectively.

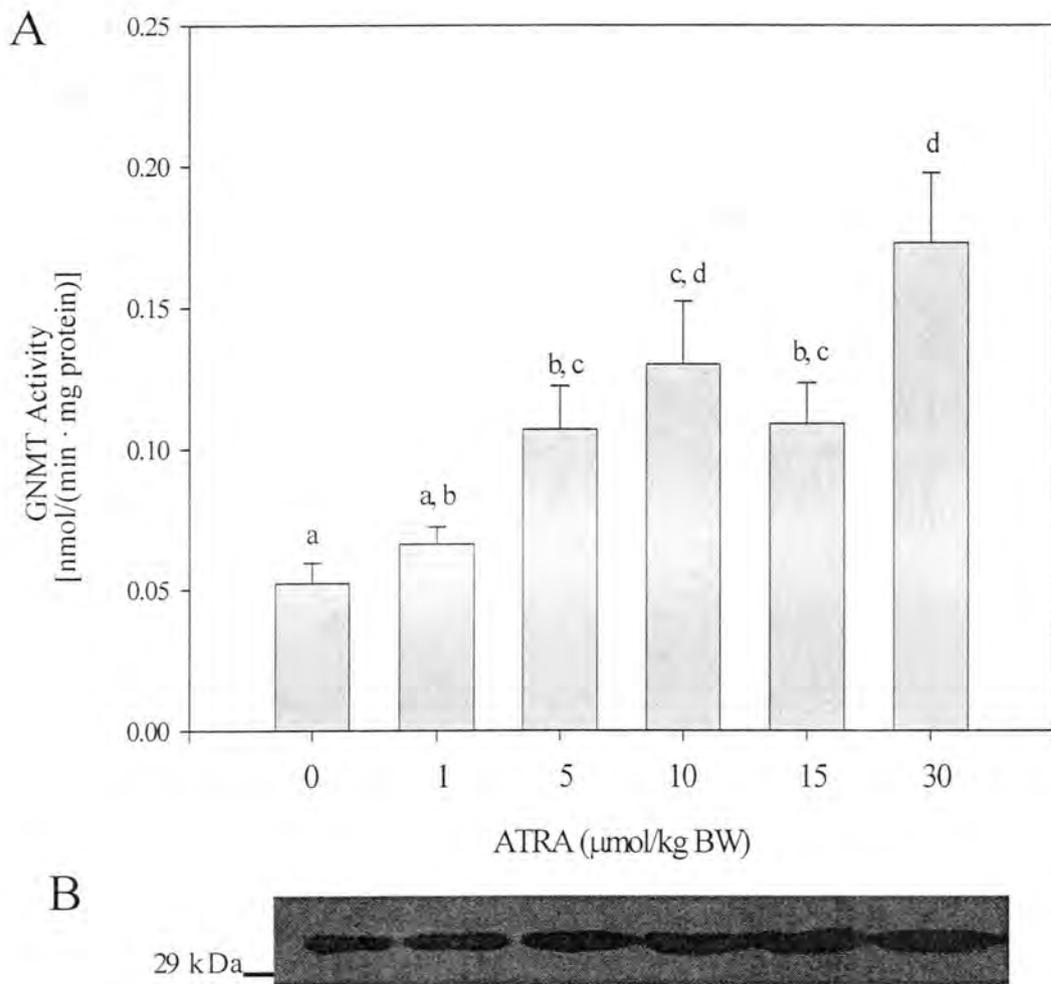


FIGURE 2.2 Dose-dependent induction of hepatic glycine *N*-methyltransferase (GNMT) by all-*trans*-retinoic acid (ATRA). Data are means \pm SEM, $n=6$. Bars without common letter denote significance ($P < 0.05$). *Panel A*, GNMT activity as a function of graded doses of ATRA given for 10 d. *Panel B*, representative immunoblot of GNMT protein abundance following 10-d treatment with various doses of ATRA.

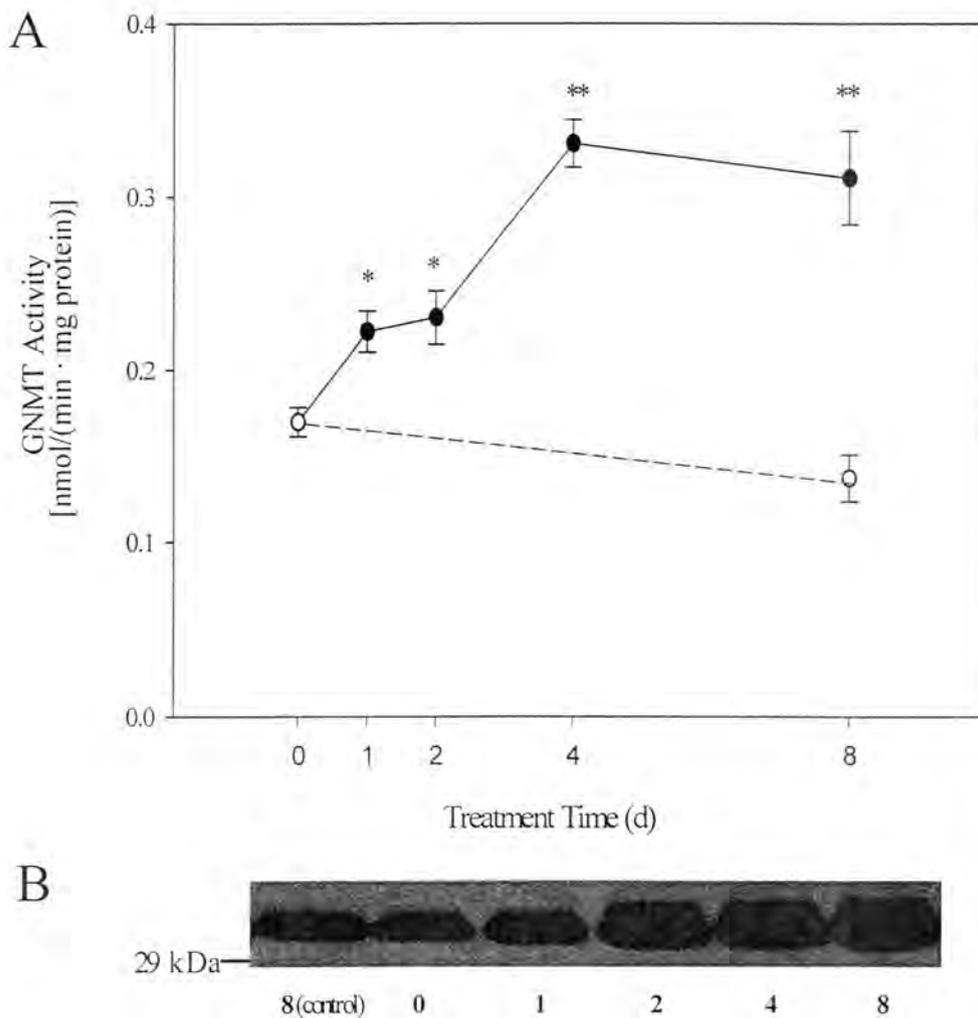


FIGURE 2.3 Time-course induction of hepatic glycine *N*-methyltransferase (GNMT) by all-trans-retinoic acid (ATRA) treatment. Data are means \pm SEM, $n = 6$. Asterisks denote values that were significantly different from control values; (* $P < 0.05$, ** $P < 0.01$). Panel A, GNMT activity as a function of treatment time with ATRA ($30 \mu\text{mol/kg BW}$). Open and closed circles represent mean values from control (vehicle-treated) and ATRA-treated rats, respectively. Panel B, representative immunoblot of GNMT protein abundance for each treatment time point.

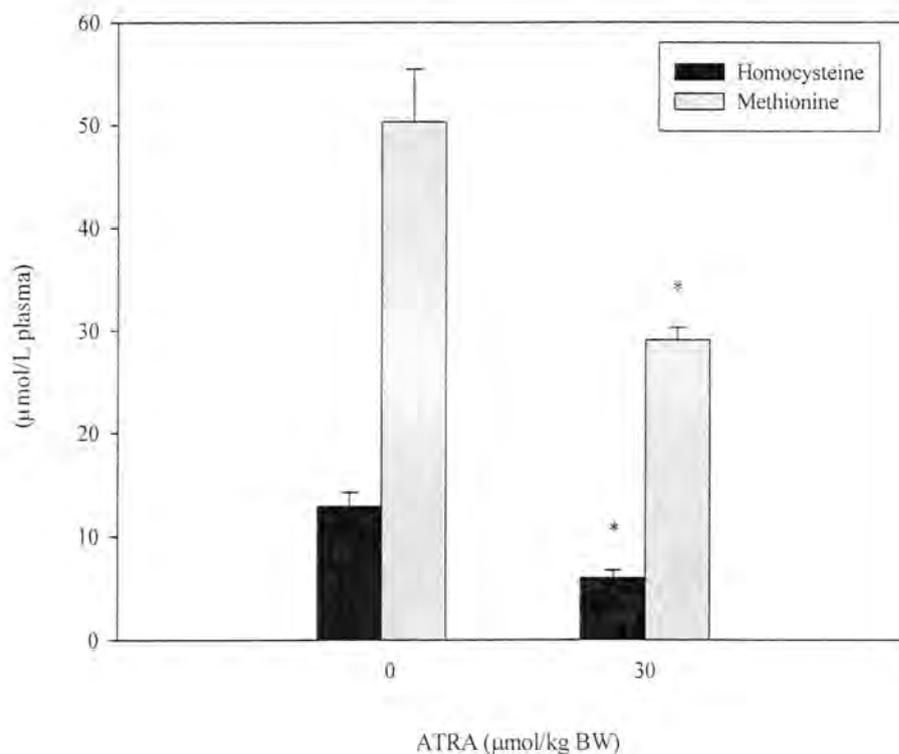


FIGURE 2.4 All-*trans*-retinoic acid (ATRA) reduces the circulating concentrations of methionine and homocysteine. Following 10-d ATRA (30 µmol/kg BW) treatment, plasma samples were analyzed for amino acid concentrations. Data are means ± SEM, n = 6. Bars with an asterisk denote significance from control values (* $P < 0.05$).

Hepatic folate coenzyme concentrations and activity of MTHFR were not affected, whereas MS activity was increased by ATRA administration.

The hepatic concentrations of THF and 5-methyl-THF were not affected by administration of ATRA (30 µmol/kg BW for 10 d) (**Table 2.1**). Likewise, the hepatic activity of MTHFR was not significantly altered following treatment with ATRA. In contrast, the hepatic activity of the homocysteine remethylation enzyme MS was elevated 40% in ATRA-treated rats compared to control activity values.

**Table 2.1 Effect of all-*trans*-retinoic acid (ATRA)¹
on folate coenzymes and enzymes**

	ATRA ($\mu\text{mol/kg BW}$)	
	0	30
	<i>nmol/g liver</i>	
THF	1.74 ± 0.20^2	1.85 ± 0.44
5-methyl-THF	1.67 ± 0.28	2.22 ± 0.46
	<i>nmol/(min · mg protein)</i>	
MTHFR	226 ± 44	357 ± 85
MS	31.8 ± 3.0	$45.5 \pm 2.4^*$

¹Abbreviations: ATRA, all-*trans*-retinoic acid; MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; THF, tetrahydrofolate.

²Data are means \pm SEM, n = 6. Value with asterisk is significantly different from control value (* $P < 0.05$).

Discussion

The availability of methyl groups for SAM-dependent transmethylation reactions is important in the production of vital biological compounds. Thus, regulation of folate and methyl group metabolism is critical to ensure optimal health, because disruption of these interrelated pathways is associated with a number of detrimental conditions (4,25,26). We have shown here and in our previous studies that the administration of retinoid compounds in large doses for 7-10 d induced GNMT, thereby compromising the methylation of important biological compounds. Of particular significance in this study was the observation that the ability of ATRA to modulate GNMT and methyl group metabolism was rapid (i.e., a single dose), and could be demonstrated at a much lower level (5 $\mu\text{mol/kg BW}$). These findings have tremendous relevance for humans that use retinoids such as ATRA (tretinoin, Vesanoïd[®]) or 13-*cis*-retinoic acid (isotretinoin, Accutane[®]) in the treatment of certain cancers and skin disorders. Recommended dosages for both of these retinoid compounds

typically range from 2-7 $\mu\text{mol}/\text{kg BW}$ and are administered for a period of 15-20 wk (27). Although we have found that 5 $\mu\text{mol}/\text{kg BW}$ was the minimum effective dose in a 10-d study, we would expect that even lower levels of retinoid compounds would be effective at perturbing GNMT and methyl group metabolism when administered for a longer time period. Taken together with our previous demonstration (14) that retinyl palmitate induced GNMT, although to a lesser extent than ATRA or 13-*cis*-retinoic acid, the unsupervised use of vitamin A supplements for a sufficient period of time may have adverse effects on methyl group metabolism as well. We are currently in the early stages of conducting research focused on the effect of chronic retinoid administration.

We have extended our previous work by examining the potential alterations expected in homocysteine metabolism as a result of retinoid administration. The concentration of homocysteine can be modulated by changes in its production (i.e., via transmethylation) and metabolism by either the transsulfuration pathway or remethylation by folate-dependent and/or folate-independent mechanisms (28). For plasma homocysteine levels, renal homocysteine metabolism plays a significant role as well. In our studies, the marked induction of GNMT would be expected to result in the accumulation of homocysteine, particularly if the capacity of the transsulfuration and/or remethylation pathways were compromised. However, we found that ATRA administration reduced circulating homocysteine levels, indicating that the metabolism of homocysteine may be enhanced. Likewise, we have reported in an earlier study that ATRA was effective at reducing elevated homocysteine concentrations to normal levels in adrenalectomized rats; however, the reduction of plasma homocysteine concentrations by ATRA in intact animals did not reach statistical significance (16). This discrepancy may reflect the difference in the experimental design, as the earlier study was only for 5 d and utilized older rats. We have found in a number of studies that older rats exhibit increased sensitivity to retinoids, as indicated by further increases in GNMT activity, but are less sensitive to the effect of retinoids on homocysteine metabolism¹.

¹ Knoblock, V.E. & Schalinske, K.L., unpublished data.

In an attempt to begin to address how retinoid administration modulates homocysteine metabolism, we examined the hepatic folate-dependent remethylation of homocysteine as a possible mechanism. Although the hepatic concentration of 5-methyl-THF and the activity of MTHFR were not statistically altered, the elevated activity of MS clearly indicates that remethylation of homocysteine by the one-carbon pool was enhanced in ATRA-treated rats. Thus, it appears that the folate-dependent one-carbon pool attempts to compensate for the retinoid-mediated loss of methyl groups by increasing their supply. Additional *in vivo* regulation may contribute to homocysteine remethylation as well. For example, allosteric factors such as the concentration of SAM plays a significant role in the regulation of MTHFR by inhibiting its enzymatic activity (5,6). We have found that retinoids reduce hepatic SAM concentrations (29,30); thus, an elevation in the endogenous MTHFR would be expected to further contribute to homocysteine remethylation.

Similar to homocysteine, it appears that ATRA treatment increased the catabolism of methionine, as indicated by the reduction in circulating methionine levels. This observation supports our earlier work and others that retinoid compounds have the ability to enhance methionine catabolism under both normal and excess dietary methionine conditions (29-32). This finding also underscores the implication that the effect of retinoids on hepatic sulfur amino acid metabolism may have an impact on reducing their availability for other tissues/cells.

It is not clear how retinoid administration may alter the metabolism of homocysteine in humans. Schulpis *et al.* (33) reported an inability to catabolize a methionine load and increased homocysteine levels in adults that received 0.5 mg/kg BW (i.e., 1.7 μ mol/kg BW) isotretinoin (13-*cis*-retinoic acid) for 45 d; however, this may reflect the hepatotoxicity associated with long-term isotretinoin usage exhibited by these patients. In our rat studies using large (30 μ mol/kg BW) doses of ATRA administered for 10 d, serum liver enzymes were not elevated (data not shown). It will be important in future research to determine the potential impact of these findings on humans. Extrapolation of rat studies focused on factors that modulate homocysteine metabolism to the human situation has met with conflicting results. For example, the alterations seen in homocysteine metabolism as a function of diabetes appears to be similar to that observed in humans (34), whereas thyroid status has

opposing effects on homocysteine metabolism when comparing the findings from rat studies to those reported from human research (35).

In summary, we have shown that the administration of ATRA, in doses equivalent to those used clinically, was effective at altering hepatic GNMT activity and potentially perturbing methyl group metabolism. Moreover, this effect was rapid and resulted in increased metabolism of methionine and homocysteine, the latter due, at least in part, to enhanced folate-dependent remethylation. These findings may have a significant impact on a number of individuals that may be exposed to retinoid usage. It has recently been reported that the use of isotretinoin has increased 2.5-fold in the last 8 years, averaging 800,000 new prescriptions per year (36). Moreover, it would be anticipated that a significant percentage of these individuals may have suboptimal nutritional status, polymorphisms of key enzymes, or a physiological condition that further compromises folate function and methyl group metabolism. We have recently shown that the combination of glucocorticoid treatment/diabetes and retinoid administration exerts an additive effect on disrupting methyl group metabolism (16).

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CHAPTER 3. GENERAL CONCLUSIONS

Summary

The role of the folate-dependent one-carbon pool and methyl group metabolism is important in health and disruptions to the interrelated cycles are implicated in the development of cardiovascular disease, cancer, and neural tube defects. Folate is needed for normal cellular function and perturbation from genetic enzyme polymorphisms, nutritional deficiencies, drug therapies or hormonal fluctuations can affect health, whether it is in the form of cardiovascular disease from excess homocysteine concentration, or the disruption of DNA synthesis leading to tumor formation.

One such perturbation to methyl group metabolism has come in the form of synthetic retinoids that are administered in the field of dermatology and the treatment of cancer. These vitamin A derivatives are useful but may come at the expense of folate and methyl group metabolism. Current research in this area is trying to elucidate the connection between vitamin A and methyl group metabolism and ultimately, the consequences involved in the administration of these synthetic retinoids as well as the dietary intake of retinoids in high doses.

The studies described in Chapter 2 demonstrate the ability of all-*trans*-retinoic acid (ATRA) on methionine metabolism. ATRA administered in doses similar to those seen in clinical dermatology and for a shorter time increased the enzyme activity of glycine *N*-methyltransferase (GNMT) and protein abundance and decreased plasma homocysteine and methionine concentrations, the result of enhanced homocysteine metabolism. The increased GNMT activity decreased the S-adenosylmethionine-dependent creatine production that was reflected by a decrease in urinary creatinine (Appendix A). Transsulfuration metabolites from homocysteine were also affected and this was demonstrated by a decrease in both urinary inorganic sulfates and plasma glutathione.

Our lab has shown that in addition to ATRA, other factors affect GNMT activity, leading to disruption of methyl group metabolism. Areas we have studied include dietary protein levels, glucocorticoid treatment, age and gender influence, and altered thyroid

function. Alterations in folate-dependent one-carbon and methyl group metabolism are driving our research with a goal of determining how modulation of these pathways affects overall health. One must be cautious when extrapolating this data from rats to humans but this research can give scientists valuable insight on disorders where these pathways are involved.

Recommendations for Future Research

Understanding the folate-dependent one-carbon pool and methyl group metabolism has taken a new turn as science has evolved with molecular techniques. Gene sequencing and DNA techniques aid in determining the characteristics of the enzymes and cofactors involved in these important pathways. The involvement of methyl group metabolism in diabetes has only recently been determined and the next 10 years of research in this area may assist in elucidating the etiology of this debilitating disease. The strong correlation of homocysteine and folate to cardiovascular disease has launched a multitude of studies looking at preventive lifestyle factors as well as the genetic influence that can cause this life-threatening disease. Identifying plasma homocysteine levels as a marker may help in the prevention of cardiovascular disease and aid researchers with other diseases in which coenzymes of methyl group metabolism may be involved.

In regards to the application of retinoids in the clinical setting, further research could be applied to examine the metabolites within methyl group metabolism as well as enzymes and coenzymes of rats treated with ATRA at low concentrations for a time frame (4-5 months) similar to the clinical dosing regime. This would clarify the intermediate effect ATRA has on methyl group metabolism in humans are undergoing retinoid therapy. Using the same study design but continuing the life span of the rat to detect tumor formation would clarify if there were a carcinogenic effect from the DNA hypomethylation that occurs with ATRA administration. Another step to take in researching retinoid therapy would be a clinical study to correlate the animal data to human application. Patients undergoing retinoid therapy could have blood samples taken for the analysis of red blood cell folate levels, lymphocyte DNA, and plasma homocysteine and methionine would help scientists

understand the relationship between retinoids and the folate-dependent one-carbon pool and methyl group metabolism.

APPENDIX A. TRANSMETHYLATION AND TRANSULFURATION PRODUCTS AFFECTED BY ALL-*TRANS*-RETINOIC ACID TREATMENT IN RATS

Introduction

The creatine-creatine phosphate system plays an important role in storing energy and the transmission of energy when needed. According to Stöcker-Ipsiroglu (1), creatine is only produced in the liver and pancreas and transported to the brain and muscles where it is utilized by creatine kinase and ATP/ADP. Guanidinoacetate is the precursor of creatine and is methylated via the enzyme guanidinoacetate methyltransferase and consumes ~75% of the S-adenosylmethionine (SAM)-dependent methylation reactions in the liver. After utilization of this energy source, creatine is nonenzymatically cycled to creatinine and is excreted in the urine. Daily excretion of creatinine is directly proportional to total body creatine and can be helpful in diagnosis of creatine-deficient states.

Another metabolite measured in the urine is inorganic sulfate, which is an end product of the transsulfuration pathway. Homocysteine is irreversibly converted to cystathionine, which is further catabolized to ammonium, α -ketobutyrate, and cysteine. Cysteine can be involved in the production of glutathione. Stipanuk *et al.* (2) showed that low cysteine availability increased the utilization of glutathione, avoiding decreased levels. High cysteine levels favor the utilization of sulfate and taurine, removing the cell of excess cysteine. These sulfated products are excreted in the urine when no longer biologically needed. Plasma glutathione was measured as well as inorganic sulfates to determine if homocysteine catabolism induced by elevated activity of glycine *N*-methyltransferase (GNMT) altered these transsulfuration products.

Materials and Methods

Animals and diet.

This study's design involving the use and care of animals is the same one in Chapter 2 and is described in detail on pp. 32-33. In regards to the samples taken for analysis in this appendix, rats were anesthetized at the end of the treatment period and whole blood was obtained via cardiac puncture for the determination of plasma glutathione and creatinine. Rats from the 0 and 30 $\mu\text{mol/kg}$ BW all-*trans*-retinoic acid (ATRA) groups were placed in metabolic cages 24 hours before they were killed to collect urine for the analysis of urinary inorganic sulfates and creatinine.

Creatinine determination.

Urinary creatinine concentrations were measured spectrophotometrically using a commercial kit (Sigma, St. Louis, MO) with slight alterations. Using urine from the same pool as the inorganic sulfate assay, 3.0 mL alkaline picrate solution (50 mL creatinine color reagent and 10 mL 1.0 N sodium hydroxide) was added to 100 μL of urine. After being vortexed, an aliquot of 2.0 mL of the mixture was transferred to a cuvette and read at 500 nm. After an 8 min incubation period, 60.6 μL acid reagent was added, vortexed and read after another incubation period of 5 min. Samples were analyzed by taking the difference between the two measurements and using the standard curve previously generated. Assay was performed on 0 and 30 $\mu\text{mol kg/BW}$ ATRA.

Inorganic sulfate determination.

Urine levels from 0 and 30 $\mu\text{mol/kg}$ BW ATRA were brought up to a total volume of 22 mL and stored at -20°C until analysis. A 50 μL aliquot was used to determine inorganic sulfate levels according to the method of Lundquist *et al.* (3). A solution consisting of 1.0 mL HCl (0.5 mol/L) and 1.0 mL stabilizing agent [9.77 g barium chloride (0.04 mol/L), 150 g polyethylene glycol 8000, and 200 μL sodium sulfate (50 mmol/L) into 1.0 L water] was added to the aliquot in duplicate, vortexed and read after 5 min incubation at 600 nm on a spectrophotometer.

Glutathione analysis.

Plasma glutathione concentrations were determined according to the method of Ubbink *et al.* (4). In brief, heparinized blood from the cardiac puncture was immediately centrifuged ($3800 \times g$ for 6 min) and plasma samples were stored at -70°C until derivatization. For derivatization, N-acetylcysteine (1 mmol/L) was added as an internal standard to 300 μL of plasma and 10% tributylphosphine in dimethylformamide solution was added followed by incubation at 4°C for 30 min. The reaction was stopped with 10% trichloroacetic acid-1.0 mmol/L EDTA and following centrifugation at $1000\times g$ for 5 min, the supernatant was added to a solution containing borate buffer (0.125 mol/L, pH 9.5), sodium hydroxide (1.55 mol/L), and 4-fluoro-7-sulfobenzofurazan (1 mg/mL). Filtered samples were analyzed by HPLC using fluorometric detection and a mobile phase consisting of 96% potassium monophosphate (0.1 mol/L, pH 2.1) buffer and 4% acetonitrile.

Statistical analysis.

The mean values of each treatment group were subjected to a one-way ANOVA (5). If the ANOVA was significant ($P < 0.05$), the means were compared using Fisher's least significant difference procedure. T-test was used in the analysis of plasma glutathione values.

Results

Transsulfuration metabolites were decreased by ATRA administration.

The transsulfuration metabolites glutathione and inorganic sulfates were affected by ATRA administration (30 $\mu\text{mol/kg}$ BW for 10 d) (**Table A.1**). Plasma glutathione was significantly lower ($P = 0.022$) in rats treated with ATRA. Urinary inorganic sulfate levels were 21% lower in rats treated with ATRA compared to control rats that were treated with vehicle, corn oil.

Plasma creatinine concentrations were not affected whereas urinary creatinine concentrations were lower in ATRA-treated rats.

There was no difference in plasma creatinine in control versus rats treated with ATRA. The administration of ATRA to rats caused a 30% decrease in urinary creatinine (0.39 mg/24 h) compared to the control group(0.56 mg/24 h) (Table A.1).

Table A.1 Transsulfuration and transmethylation metabolites

Metabolite	ATRA ¹ ($\mu\text{mol/kg BW}$)	
	0	30
Glutathione ($\mu\text{mol/L plasma}$)	21.5 \pm 2.1	15.3 \pm 0.8 ^{# 2}
Urinary Sulfates ($\mu\text{mol/24h}$)	0.38 \pm 0.03	0.30 \pm 0.02*
Urinary Creatinine (mg/24h)	0.56 \pm 0.05	0.39 \pm 0.04*
Creatinine ($\mu\text{mol/L plasma}$)	81.9 \pm 8.9	77.9 \pm 7.5

¹ Abbreviations: all-*trans*-retinoic acid, ATRA.

² Data are means \pm SEM, n=6; # represent significance in t-test using 0 and 30 ATRA ($\mu\text{mol/kg BW}$) groups ($P < 0.05$); * represents significance in ANOVA ($P < 0.05$).

Discussion

The impact of ATRA on methyl group metabolism has been demonstrated in Chapter 2; however, the subsequent decrease in metabolites from this pathway was not shown. The administration of ATRA in a pharmacological dose (30 $\mu\text{mol/kg BW}$) for 10 days had a profound effect on products that arise from the SAM-dependent transmethylation reaction and the catabolism of homocysteine. Creatine is an important carrier of energy and is primarily produced in the liver. Its catabolism produces creatinine, which is then excreted from the body. This can provide researchers with a valuable test to measure creatine stores.

There are numerous methyltransferases available to utilize the methyl group SAM releases to produce important biological products. Another methyltransferase, glycine *N*-methyltransferase (GNMT) is present for a regulatory need of maintaining the transmethylation potential. The administration of ATRA is known to induce GNMT activity and cause DNA hypomethylation, another product formed from the transmethylation reaction (6,7). The increase in GNMT activity appears to prevent other methyltransferases from methylating more important biological products (i.e., DNA, creatine).

Additionally, homocysteine metabolism was perturbed by ATRA administration. Chapter 2 showed the decrease in plasma homocysteine and this is reflected in the decrease in plasma glutathione. Another end-product from homocysteine catabolism is inorganic sulfate and was also decreased in the urine after the administration of ATRA. These results, along with those conveyed in Chapter 2, can be examined together in the analysis of ATRA's impact and what the overall health consequences may be to those who use retinoids in high doses clinically or in a supplementation.

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APPENDIX B. INCREASED PROTEIN INTAKE INDUCES GLYCINE N-METHYLTRANSFERASE ACTIVITY IN RATS TREATED WITH ALL-TRANS-RETINOIC ACID

Introduction

Methionine is an integral part of methyl group metabolism and the pathway is often referred to as methionine metabolism. Methionine favors conversion to S-adenosylmethionine (SAM), an activated molecule that can regulate enzyme activity concurrent to its concentration (1). The enzymes methionine synthase (MS) and betaine:homocysteine methyltransferase (BHMT) respond to fluctuations in methionine concentration, as shown in Table 1.1 (2). A deficiency of methionine is handled efficiently as inducing enzyme activity within the methionine pathway, resulting from decreased SAM concentration by increasing activity and remethylating homocysteine (3). If the methionine concentration increases beyond the metabolic needs of SAM, the transsulfuration pathway lowers the methionine level via the enzyme cystathionine β -synthase (CBS) to irreversibly catabolize homocysteine (4,5). Finkelstein *et al.* (1) reported that when a high protein diet is ingested followed by a low protein diet, the conversion of homocysteine to cystathionine, the first product in the transsulfuration pathway drops from 70% to 10%. The same phenomenon occurs when high doses of SAM and S-adenosylhomocysteine (SAH) are given, causing homocysteine catabolism to cystathionine to increase from 39% to 82%. Another enzyme that is affected by an increase in protein intake is glycine N-methyltransferase (GNMT), which is viewed as a regulatory enzyme that is active in times of excess SAM levels. GNMT catalyzes the donation of the SAM methyl group to glycine to produce sarcosine, a molecule with no known biological function (6). Ingestion of a 3% methionine diet results in an increase in hepatic GNMT activity to rid the cell of excess methyl groups available from increased SAM concentration (7).

Because of its overall impact on the body, the homeostasis of methyl group metabolism is important to study to determine what occurs when outside factors are added. Identifying the disturbances that take place when retinoids are administered has been

established. It is important to test the outcome that additional factors have on the pathway. The following results show the alterations of a 20% casein diet along with the administration of either vehicle (corn oil) or 30 μmol all-*trans*-retinoic acid (ATRA)/kg BW in the rat model. These treatment groups were compared to control groups fed a diet consisting of 10% casein, a level of protein needed for basal metabolism, combined with the administration of ATRA or the vehicle.

Materials and Methods

Chemicals and reagents.

Reagents were obtained from the following: *S*-adenosyl-*L*-[*methyl*- ^3H]methionine, New England Nuclear (Boston, MA); phenylmethylsulfonyl fluoride and ATRA, Calbiochem (La Jolla, CA); and *S*-adenosyl-*L*-methionine, Sigma Chemical (St. Louis, MO).

Animals and diet.

All animal experiments were approved by and conducted in accordance with Iowa State University Laboratory Animal Resources Guidelines. For all studies, male Sprague-Dawley (Harlan Sprague-Dawley, Indianapolis, IN) rats (50-74 g) were housed in plastic cages in a room with a 12-h light: dark cycle. Rats were acclimated to the control diet containing 10% methionine from casein (8) with the exception of vitamin/mineral mix 93 for 7 d and the oral administration of corn oil for 6 d. Half of the rats were fed a 20% methionine diet by increasing the casein concentration in the diet. ATRA was administered to the treatment group as 30 $\mu\text{mol}/\text{kg}$ BW using corn oil as the vehicle for a total of 8 d. At the end of the treatment period, rats were anesthetized and whole blood was obtained via cardiac puncture for determination of plasma homocysteine concentrations. Liver samples were obtained for determining the enzymatic activity of GNMT and folate coenzyme concentrations, as well as lipid and triglyceride content.

Measurement of GNMT activity.

GNMT enzymatic activity was assayed as described by Cook and Wagner (6) with minor modifications. Portions of liver were homogenized in three volumes of ice-cold phosphate buffered (10 mmol/L, pH 7.0) sucrose (0.25 mol/L) containing 1 mmol/L EDTA, 1 mmol/L sodium azide, and 0.1 mmol/L phenylmethylsulfonylfluoride. After centrifugation ($20,000 \times g$ for 30 min), supernatants were stored with 2-mercaptoethanol (10 mmol/L) at $-70^{\circ} C$. The assay contained 1 mmol/L Tris buffer (pH 9.0), 5 mmol/L dithiothreitol, 10 mmol/L glycine, and 1 mmol/L *S*-adenosyl-*L*-[methyl- 3H]methionine (47.7 kBq/ μ mol) and the reaction was initiated with 250 μ g of the protein supernatant. For the determination of total soluble protein in the tissue extract, a commercial kit (Coomassie Plus, Pierce, Rockford, IL) based on the method of Bradford (9) was used with bovine serum albumin as a standard.

Lipid detection.

Lipids were analyzed according to Folch *et al.* (10). 1.0 g of liver tissue was extracted and wrapped in aluminum foil and stored at $-70^{\circ} C$ until analysis. After warming to room temp, liver samples were weighed, added to 10.0 mL ice-cold 2:1 chloroform/methanol, and homogenized. Homogenate was then applied to a moistened Whatman filter placed in a funnel/graduated cylinder apparatus to be filtered. Additional chloroform/methanol solution was added to the sample to reach 20 mL. The funnels were removed and 4.0 mL calcium chloride (0.05%) was added. Enclosed graduated cylinders were inverted 5 times and allowed to set overnight. Extraction occurred and the upper phase was pipetted off carefully as to not disrupt the interface. 1.0 mL of upper phase solvent (8:4:3 containing chloroform: methanol: 0.04% calcium chloride) was added and gently mixed by rotation before the top phase was pipetted off. This procedure was performed 2 more times and 5 drops of methanol were applied afterwards to remove the interface. The chloroform/methanol mixture was added to bring the solution up to 20 mL and gently mixed. Three aluminum cups per sample were preweighed and 5.0 mL of solution were added to each. Solution was allowed to evaporate overnight and aluminum cup was weighed to measure precipitate. Difference between initial cup weights and the precipitate represented

¼ of total lipids/g liver. Leftover solution was stored at room temp for the analysis of triglycerides.

Triglyceride detection.

Triglycerides were measured spectrophotometrically using a commercial kit (Sigma, St. Louis, MO). Briefly, 100 µL solution from the lipid assay was added to a test tube and the solvent was evaporated in a vacuum state. The reagents the kit provided were added, tubes were vortexed, and incubated for 10 min at 30 ° C. The solution was transferred to a cuvette and read at 540 nm. The absorbance was analyzed using the standard curve generated earlier and divided by the initial liver weight from the lipid assay to find mg triglyceride/g liver.

Statistical analysis.

The mean values of each treatment group were subjected to a two-way ANOVA (11). If the ANOVA was significant ($P < 0.05$), the means were compared using Fisher's least significant difference procedure and analyzed by one-way ANOVA to identify significance within groups.

Results

ATRA induced hepatic GNMT and was exacerbated by the 20% casein diet.

GNMT activity was significantly higher in rats treated with ATRA as shown in **Figure B.1**. The application of the 20% casein diet plus ATRA (30 µmol/kg BW) amplified GNMT activity 36% compared to control diet treated with ATRA. The GNMT activity of the 20% casein diet ATRA-treated group was 184% higher compared to the group fed the 20% casein diet dosed with 0 µmol/kg BW ATRA.

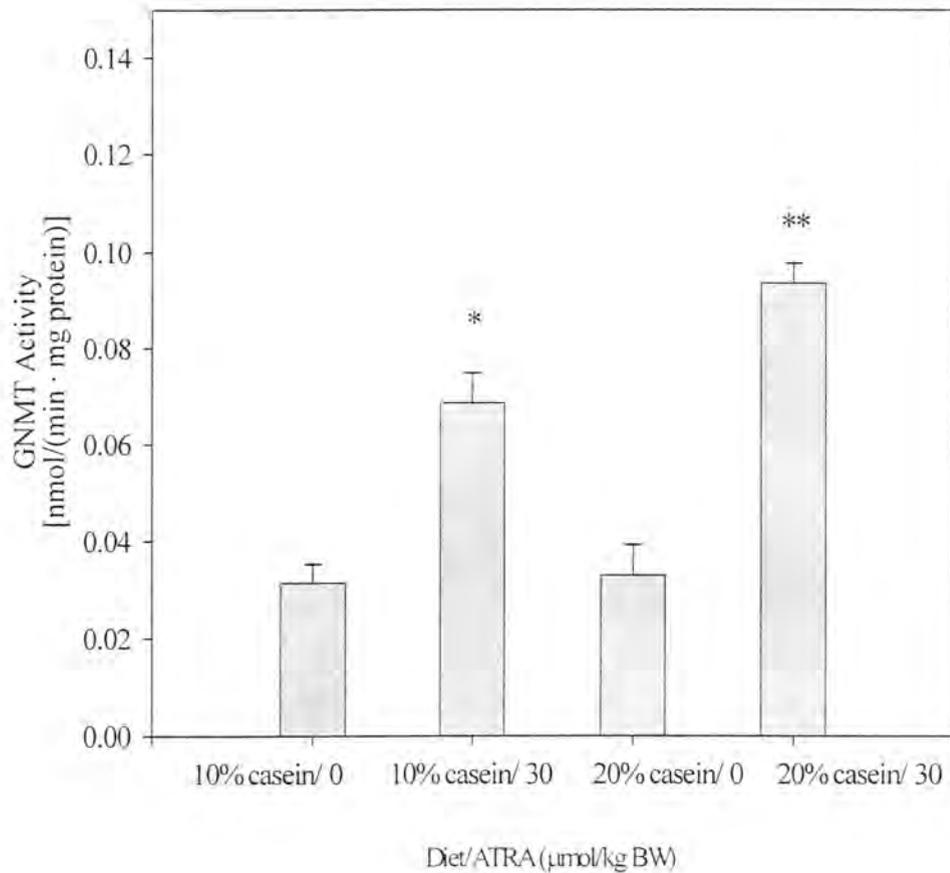


Figure B.1 Induction of GNMT in rats treated with ATRA and/or 20% casein diet. Data are means \pm SEM, $n = 5$. Bars with asterisks differ. (* $P < 0.05$, ** $P < 0.01$).

20% casein diet had a protective effect from increased hepatic lipid and triglyceride levels in rats treated with ATRA compared to ATRA-treated rats on the control diet.

Rats treated with ATRA (30 $\mu\text{mol/kg BW}$) experienced significantly higher hepatic lipids than control group (**Figure B.2**). This effect was not seen in ATRA-treated rats that consumed the 20% casein diet. The ATRA-treated group on the control diet had 230% higher hepatic triglyceride content compared to the ATRA-treated group on the 20% casein diet.

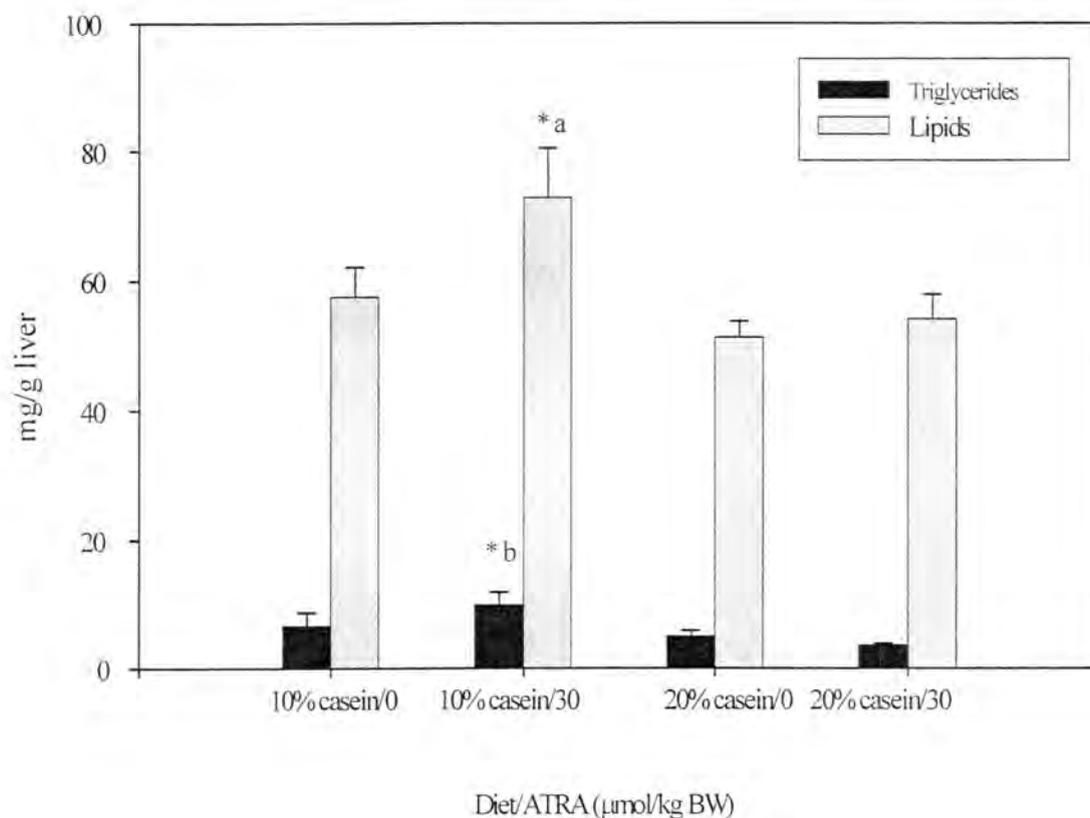


Figure B.2 The effect of ATRA and 20% casein diet on rats. ATRA increased liver lipids and triglycerides on rats fed the 10% casein diet but did not affect those on the 20% casein diet. ^a denotes significance compared to all other groups. ^b denotes significance compared only the 20% casein diet. Data are means \pm SEM, $n = 5$. Bars with asterisks differ, ($* P < 0.05$).

Discussion

GNMT is an important regulatory enzyme in methyl group metabolism. We have shown that the application of ATRA (30 $\mu\text{mol/kg BW}$) to rats induces its activity and abundance while ingesting a control diet (10% casein) (12,13). When the protein amount in

the diet increases (20% casein), there appears to be an additive effect with ATRA on GNMT activity. The increase in protein correlates to an increase in overall methionine, which corresponds to increased cellular methionine concentration within methyl group metabolism. The increased amount of methionine may have led to higher SAM concentration and a need for GNMT to dispose of excess methyl groups. The control diet includes 10% casein and added methionine to produce a total methionine content of 0.58%; additionally, the 20% casein diet used in this study has a total methionine content of 0.86%. Rowling *et al.* (14) found an increase in GNMT activity from a 1.0% methionine diet while the 0.5% methionine diet did not affect the activity.

The two diets used in this study did not alter GNMT activity when ATRA was not administered (vehicle corn oil was given). However, GNMT activity from the 20% casein/ATRA treatment group was 36% higher than the group receiving the 10% casein/ATRA. These results indicate the additive effect of two perturbations to methyl group metabolism and the subsequent increase in GNMT activity.

The addition of increased methionine in the diet appears to protect the liver from ATRA-mediated triglyceride and hepatic lipid increases. Liver lipids were lowered in ATRA-treated rats fed the 20% casein diet compared to those treated with ATRA on the control diet. Triglyceride levels of ATRA-treated rats fed the control diet were significantly higher than rats fed the 20% casein diet. The impact of increased protein intake on methyl group metabolism is important to document. In cases of genetic polymorphisms of enzymes and other nutritional deficiencies, increased protein may have a detrimental effect in the methionine metabolism. However, it does have a protective effect against fatty liver, which is a side effect of ATRA administration (15) and is important in the area of hepatic cancer.

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