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Modulation of methyl group metabolism by diabetes and retinoids: implications for complications associated with a diabetic state

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Modulation of methyl group metabolism by diabetes and retinoids: Implications for complications associated with a diabetic state

by

Kristin Marie Nieman

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

Major: Nutrition

Program of Study Committee:
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Ames, Iowa

2004

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This is to certify that the master's thesis of
Kristin Marie Nieman
has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

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LIST OF ABBREVIATIONS

BHMT	betaine-homocysteine <i>S</i> -methyltransferase
CBS	cystathionine β -synthase
CRA	13- <i>cis</i> -retinoic acid
CVD	cardiovascular disease
DEX	dexamethasone
DFE	dietary folate equivalent
DMG	dimethylglycine
DRI	dietary reference intake
dUMP	deoxyuridine monophosphate
EAR	estimated average requirement
GNMT	glycine <i>N</i> -methyltransferase
HPLC	high pressure liquid chromatography
MAT	methionine adenosyltransferase
MS	methionine synthase
MTHFR	5,10-methylenetetrahydrofolate reductase
NTDs	neural tube defects
RA	all- <i>trans</i> -retinoic acid
RDA	recommended dietary allowance
SAM	<i>S</i> -adenosylmethionine
SAH	<i>S</i> -adenosylhomocysteine
SAHH	<i>S</i> -adenosylhomocysteine hydrolase
SHMT	serine hydroxymethyltransferase
STZ	streptozotocin
THF	tetrahydrofolate
TS	thymidylate synthase
UL	upper limit
5-CH ₃ THF	5-methyltetrahydrofolate
5,10-CH ₂ THF	5,10-methylenetetrahydrofolate
10-CHO-THF	10-formyltetrahydrofolate

ABSTRACT

Regulation of folate and methyl group metabolism is essential in the prevention of a number of pathologies including vascular disease, cancer, and neural tube defects.

Understanding the control of the enzyme glycine *N*-methyltransferase (GNMT) and other key enzymes in methyl group metabolism is critical for disease prevention. Both nutritional and hormonal factors have been identified as having the ability to modulate folate, methyl group, and homocysteine metabolism. Specifically, both glucocorticoids and/or all-*trans*-retinoic acid (RA) have shown to induce active GNMT protein. This study was conducted to determine how a diabetic state, alone and in combination with RA-treatment, altered methyl group metabolism. GNMT activity increased 87% and 148% with diabetes and RA treatment, respectively. This induction of GNMT activity by diabetes and RA was also reflected in the abundance of the protein. In cell culture studies, pretreatment with insulin prevented the induction of GNMT by RA and dexamethasone. A decrease in homocysteine concentrations was exhibited in diabetic rats, possibly as a result of a 38% increase in the abundance of the cystathionine β -synthase (CBS) which commits homocysteine to catabolism. Treatment of diabetic rats with RA prevented the induction of CBS. A diabetic state and RA-treatment seemed to rely on different remethylation mechanisms. Folate-independent remethylation enzyme betaine-homocysteine *S*-methyltransferase (BHMT) increased in a diabetic state. Conversely, RA administration caused an increase in activity of the folate-dependent enzyme, methionine synthase (MS), but did not reach significance. These results indicate that both a diabetic condition and RA treatment cause significant alterations in the metabolism of methyl groups and homocysteine, a finding that may have implications for diabetics and the potential for complications associated with retinoid use.

CHAPTER 1 - GENERAL INTRODUCTION

Thesis Organization

The contents of this thesis are organized into several chapters. Chapter 1 is an introduction giving the reader an idea of the research questions the author focused on. A broad review of the literature constitutes Chapter 2 including information that is pertinent to the author's research in folate and methyl group metabolism. Chapter 3 contains research submitted to the *Journal of Biochemistry* exploring the factors, diabetes and retinoid treatment, in folate and methyl group metabolism. General conclusions and ideas for future research are incorporated into chapter 4, followed by a complete list of the literature referenced throughout this thesis.

Description of the Research Questions

The interrelated folate, methyl group, and homocysteine pathways function to carry and activate one-carbon units in the formation of essential biological compounds throughout the body. Specifically, these mechanisms are vital in the production of nucleic acids, proteins, phospholipids, and neurotransmitters. Therefore interruption of these pathways can result in a number of diseases including cancer, vascular disease, and neural tube defects.

A number of factors have been identified as having the ability to alter folate, methyl group, and homocysteine metabolism. Our laboratory has demonstrated that the vitamin A derivative all-*trans*-retinoic acid (RA) disrupts methyl group metabolism by activating the enzyme glycine *N*-methyltransferase (GNMT) as well as increasing protein abundance *in vitro* and *in vivo*, which in turn leads to loss of methyl groups for SAM-dependent transmethylation reactions (Rowling and Schalinske 2001; Rowling and Schalinske 2003).

Moreover treatment with RA has shown to cause hypomethylation of DNA (Rowling *et al.*, 2002). RA has also been indicated in the transcriptional induction of key enzymes in the gluconeogenic pathway including phosphoenolpyruvate carboxykinase (Pan *et al.*, 1990; Shin and McGrane 1997).

Several studies have also reported compounds that simulate a gluconeogenic state, including glucagon and glucocorticoids, which have the ability to modify folate, homocysteine, and methyl group metabolism. The Brosnan lab group found rats treated with glucagon exhibited elevated activities of GNMT and cystathionine β -synthase (CBS), as well as decreased plasma homocysteine concentrations indicating increased flux through the transsulfuration pathway (Jacobs *et al.*, 2001). Several studies conducted in diabetic-induced rats have also revealed similar modifications in one-carbon metabolism, whereas treatment with insulin seemed to prevent these alterations (Xue and Snoswell 1985; Jacobs *et al.*, 1998; Ratnam *et al.*, 2002). Finally, glucocorticoid administration results in the induction and activation of GNMT in cell culture and animal models (Rowling and Schalinske 2003) and increased cellular levels of CBS protein and CBS mRNA *in vitro* (Ratnam *et al.*, 2002). Moreover, Rowling and Schalinske found that glucocorticoid treatment was just as effective as RA in inducing GNMT (2003). Based on the aforementioned evidence, the goal of our study was to determine how a diabetic state and RA, alone or combined, effect methyl group metabolism.

CHAPTER 2 - LITERATURE REVIEW

Folate Metabolism

Structure and Function of Folate Compounds

Structure

Folate, a generic term for a family of essential water soluble B-vitamins, functions primarily to accept and donate one-carbon units in vital metabolic pathways throughout the body (Bailey and Gregory 1999a; Lucock and Daskalakis 2000). This family of vitamins, also known by the chemical name pteroylglutamic acid, contains both natural and synthetic forms all of which are similar in structure. The basic structure of folate moieties consists of a pteridine ring, p-aminobenzoic acid, and one or more glutamic acid residues linked by γ -carboxyl peptide bonds (**Figure 2.1A**). Folate forms differ by altering the structure in three

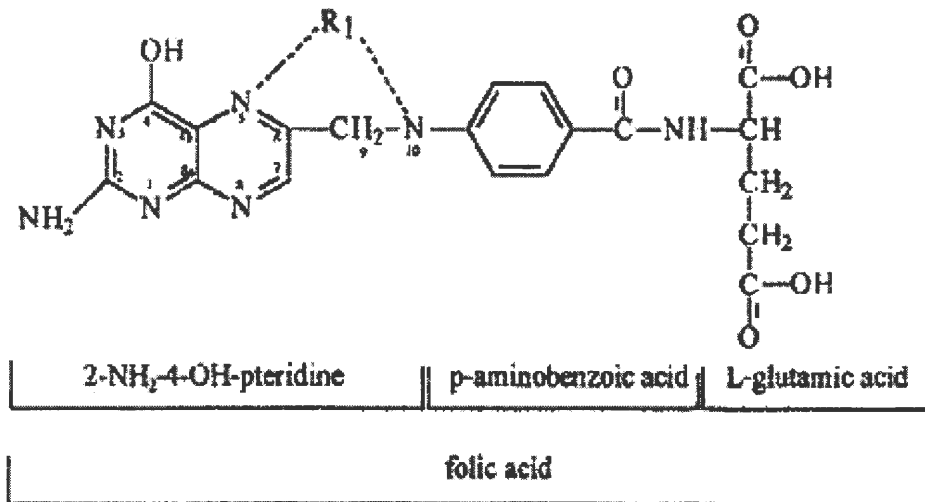


Figure 2.1A. Basic structure of folate molecules (modified from van der Put *et al.*, 2001).

locations: modifying the reduction state of the pteridine ring, adding one-carbon units at the N₅ and/or N₁₀ binding sites (**Figure 2.1B**), or varying the number of glutamic acid residues

attached. Naturally occurring folate is typically polyglutamated containing between three and seven glutamyl residues and exists most commonly as 5-methyltetrahydrofolate (5-CH₃THF) and 10-formyltetrahydrofolate (10-CHO-THF). The synthetic form of folate, folic acid used in supplements and fortified foods, is monoglutamated (van der Put *et al.*, 2001).

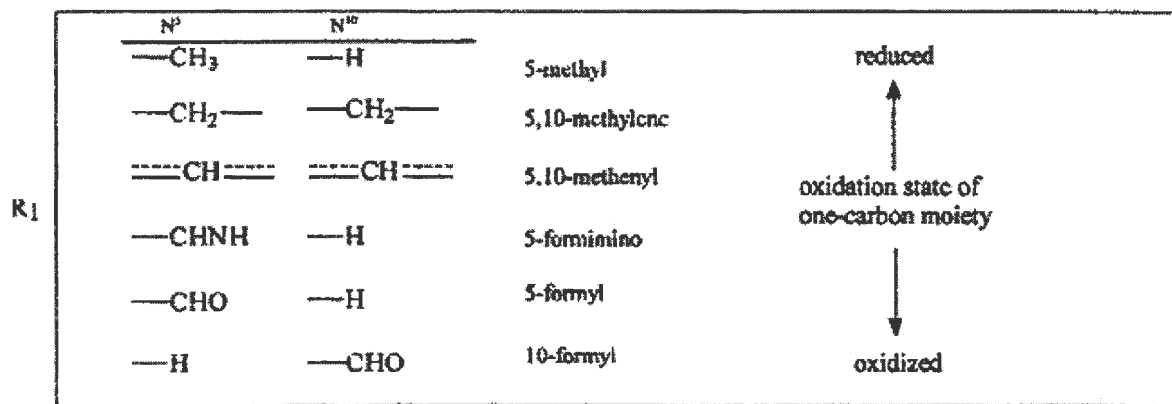


Figure 2.1B. Folate derivatives (modified from van der Put *et al.*, 2001)

Function

The donation of one-carbon units is essential in the production of nucleic acids (DNA and RNA), proteins, as well as in the metabolism of amino acids. These one-carbon units exist at various oxidation levels, as seen in figure 2.1B, and the reactions they are involved in are collectively known as folate-dependent one-carbon metabolism (Wagner 1995). The reduced folate derivative, tetrahydrofolate (THF), accepts one-carbon units at the oxidation level of formate from histidine degradation, or at the oxidation level of formaldehyde from the third carbon of serine (Shane and Stokstad 1985). However, one-carbon units can also be derived from glycine, dimethylglycine (DMG), and methylglycine (van der Put *et al.*, 2001). Specifically folate, in the form of 10-CHO-THF, is needed to donate carbon 2 and 8 in the

purine component of DNA (**Figure 2.2**). The folate derivative, 5,10-methylenetetrahydrofolate (5,10-CH₂THF), is involved in the biosynthesis of the pyrimidine building block of DNA (Shane and Stokstad 1985; Wagner 1995; Scott and Weir 1998). Aside from the role of folate in the biosynthesis of nucleic acids, one-carbon units provided by folate are used in over one hundred methyltransferase-catalyzed reactions (van der Put *et al.*, 2001) (**Figure 2.3**).

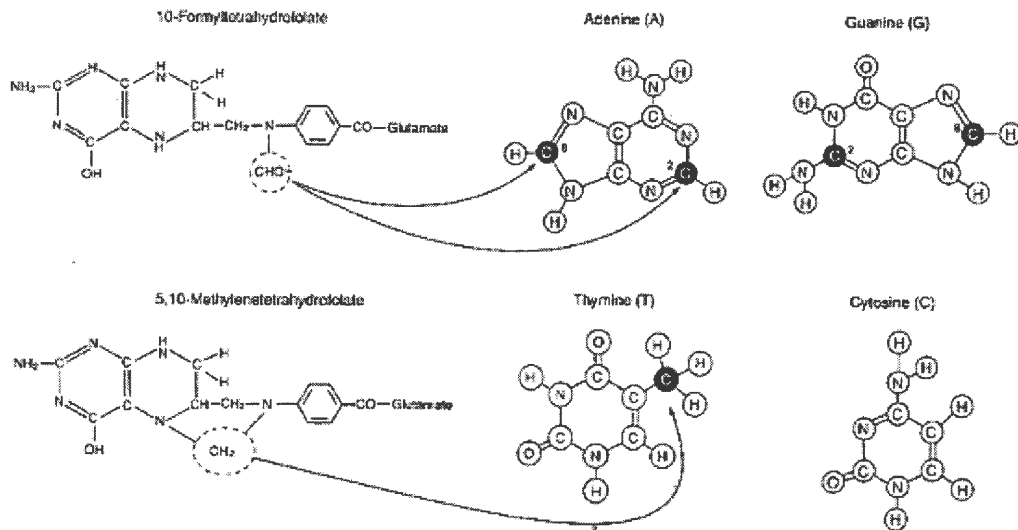


Figure 2.2. The role folate in purine and pyrimidine biosynthesis (from Scott and Weir 1998).

Folate Utilization

Digestion and Absorption

Mammals are unable to synthesize folates *de novo*, making it necessary to attain folate compounds through consumption (Shane 1995). The polyglutamated chain of naturally occurring folates must be removed before the vitamin is digested. Excess glutamate residues are hydrolyzed by the enzyme γ -glutamyl hydrolase in brush border mucosal cells of the proximal small intestine, and in turn this monoglutamated form is absorbed (McDowell

2000). Typically if the folate compound is not already in the form of 5-CH₃THF, it will be converted to this form and transported through the intestinal mucosa cell (Scott 1999). The monoglutamated synthetic form of folate is readily absorbed, primarily in the jejunum.

Transport and Storage

Following absorption, folate passes through the mucosal cell and is taken up by the liver via portal circulation (Shane 1995). In the liver, folate remains in the form 5-CH₃-THF or is otherwise converted to 10-CHO-THF and transported to peripheral tissue (McDowell 2000). The transport of folate into cells can be accomplished by one of three mechanisms: (i) unidirectional transport by membrane-bound folate-binding proteins/receptors, (ii) bidirectional internalization by carrier-mediated reduced folate carriers, (iii) or by passive

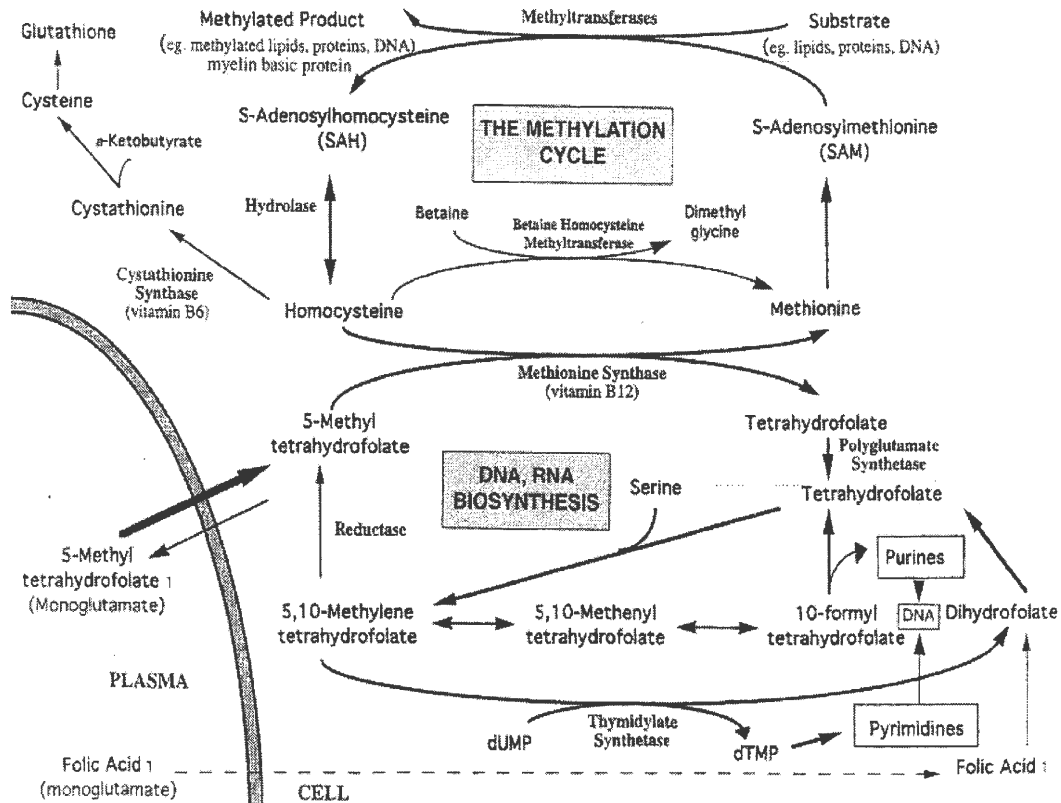


Figure 2.3. Folate and one-carbon metabolism (from Scott and Weir 1998)

diffusion (Sirotnak and Tolner 1999; Suh *et al.*, 2001). Once folate enters the cell the enzyme methionine synthase (MS) converts 5-CH₃THF to THF the favored substrate of the enzyme, folylpolyglutamate synthase. The activity of this enzyme, necessary to retain folate within the cell, results in the polyglutamylation of THF and is present in both the mitochondria and cytoplasm (Lucock and Daskalakis 2000).

Polyglutamylated folate is compartmentalized between the cytoplasm and the mitochondria (Cook and Blair 1979). Most folate in the cell is protein-bound and usually remains in their respective compartment; however the products that result from folate compounds are easily transferred between the cytoplasm and mitochondria (Lin and Shane 1994). At any time cellular concentrations of free folate are negligible due to tight-binding proteins, which also limits intracellular folate accumulation when saturated (Matherly *et al.*, 1990). The body typically stores between 5 and 10 mg of folate, half of which is found in the liver in the form of 5-CH₃THF (Herbert 1999).

Degradation and Excretion

Protein-bound folates are stable compared to free-folate, which are often depleted in the cell due to their instability. There are three mechanisms that are thought to cause the turnover of folates, specifically unbound folate: exit of folate molecules from lack of polyglutamylation, folate polyglutamates are hydrolyzed to monoglutamates by γ -glutamylhydrolase and exit the cell, or folate catabolism (Suh *et al.*, 2001). A majority of folate excretion is in feces via bile due to enterohepatic circulation most of which is reabsorbed in the intestine. However, a small portion of excreted folate can be found in urine following glomerular filtration and reabsorption of some of the folate (Herbert 1999).

Degradation of folate generates pteridines and acetaminobenzoylglutamate.

Dietary Requirements

Sources of Folate

Currently recommendations for folate intake are focused on reducing the risk of chronic disease, which means maintaining optimal one-carbon metabolism (Boushey *et al.*, 1995). Folates are present in most natural foods with fruits and vegetables being the richest sources. Specifically, spinach, brussel sprouts, potatoes, oranges, beans, and liver, are excellent sources of folate (Bailey 1995; van der Put *et al.*, 2001). However, these natural forms of folate are particularly susceptible to oxidative damage, and it is estimated that 50-95% of the folate is destroyed by cooking excessively (Herbert 1999). There are also several states in the body that can result in altered folate status and increased catabolism and in turn increases folate requirement such as pregnancy, oral contraceptive or anticonvulsant use, cancer, or chronic alcohol consumption (Suh *et al.*, 2001). It is because of this potential for folate deficiency and the risk of congenital defects as well as chronic disease associated with deficiency that the U.S. Food and Drug Administration made it mandatory for enriched grain products to include folic acid (1996). This fortification has led to a decline in the prevalence of folate deficiency-related pathologies specifically neural tube defects (Honein *et al.*, 2001).

Recommended Intake

Folate dietary requirements are expressed as recommended dietary allowances (RDA) which is derived from the estimated average requirement (EAR) as well as adjusting to meet the requirements for approximately 98% of the population. The EAR is defined as the amount needed to meet only 50% of the population (Bailey and Gregory 1999a). Given that synthetic folate is much more readily available than dietary folate, dietary reference intakes (DRIs) for folate are expressed as dietary folate equivalents (DFE). One DFE is equivalent

to 1 µg of food folate, 0.6 µg of synthetic folate from fortified food or a supplement with food, or 0.5 µg of a supplement on an empty stomach (Food and Nutrition Board 2000).

RDAs vary depending on age and state with males and females older than 14 years recommended to take in 400 µg DFE per day. The RDA declines for children less than 14 years old to between 65 and 80 µg DFE per day in infants up to 12 months old. Folate requirements increase in the case of pregnancy and lactation at 600 and 500 µg DFE per day respectively (**Table 2.1**). This elevated requirement is due to the increased need for folate in

Table 2.1
Folate dietary reference intakes¹

Group	Adequate intake	Recommended dietary allowance
	µg of DFE/d	
infants		
0-6 months	65	
7-12 months	80	
children and adolescents²		
1-3 years		150
4-8 years		200
9-13 years		300
4-18 years		400
adults²		
≥ 19 years		400
pregnant women		
all ages		600
lactating women		
all ages		500

¹ modified from Bailey and Gregory 1999

² male and female

cell division and increased clearance of folate during pregnancy, as well as the loss of folate in daily lactation following pregnancy (Bailey 1995). There are also upper limits (UL)

specified for each age group, meaning the maximum daily intake without inducing adverse effects. In children up to 8 years old the UL is 300-400 µg DFE per day and 600-800 µg DFE per day in males and females up to the age of 18 years old. The UL for adults above 19 years old increases to 1,000 µg DFE per day in both males and females regardless of pregnancy and lactation (Food and Nutrition Board 2000).

Methyl Group Metabolism

Functions and Components of the Metabolic Pathway

Transmethylation

Methyl group metabolism functions in activating methyl groups provided to over one hundred methyltransferase reactions necessary for normal growth and development. Methyl group metabolism begins with the set of reactions collectively called transmethylation which relies on a supply of the essential sulfur-containing amino acid, methionine (Finkelstein 1990). Methionine is converted to its activated form, *S*-adenosylmethionine (SAM) following adenylation. This reaction is catalyzed by the enzyme methionine adenosyltransferase (MAT) at the expense of ATP (Cantoni 1977; Chiang *et al.*, 1996; Lieber and Packer 2002). At this point SAM, the primary methyl donor in most mammals, can donate activated methyl groups to many methyltransferase reactions in the modification and synthesis of essential molecules resulting in *S*-adenosylhomocysteine (SAH) (Mudd 1963; Mason 2003). The formation of creatine from guanidinoacetate seems to constitute a major use of methyl groups (Stipanuk 1986). However most SAM-dependent transmethylation reactions are inhibited by SAH, therefore it essential to regulate the SAM to SAH ratio as well as remove SAH to preserve methyl groups (Cantoni *et al.*, 1978).

The folate-binding protein, glycine *N*-methyltransferase (GNMT), is believed to function in regulating the availability of SAM (Cook and Wagner 1984; Yeo and Wagner 1994). GNMT is found in the liver, pancreas, and kidney, and comprises approximately 1 to 3 % of all soluble hepatic cytosolic protein (Kerr 1972; Ogawa and Fujioka 1982). GNMT was found to be most abundant in the periportal region of the liver, the proximal convoluted tubules of the kidney, and the exocrine tissue of the pancreas (Yeo and Wagner 1994). This methyltransferase is an alternative mechanism for converting SAM to SAH, utilizing glycine and forming sarcosine, which currently has no known metabolic role (Kerr 1972; Heady and Kerr 1973). GNMT optimizes the SAM: SAH ratio, which is an indicator of transmethylation potential, given SAH is a potent inhibitor of most SAM-dependent methyltransferases (Yeo and Wagner 1994).

Transmethylation results in the production of homocysteine following the removal of adenosine from SAH. This exclusive metabolic pathway is catalyzed by *S*-adenosylhomocysteine hydrolase (SAHH) (Cantoni and Chiang 1980). This reaction is reversible favoring SAH production, and sways in the opposite direction if adenosine and homocysteine can be efficiently removed (de la Haba and Cantoni 1959; Cantoni and Chiang 1980) (**Figure 2.4**).

Remethylation

Two pathways exist that function to metabolize homocysteine: remethylation, recycling methyl groups in formation of methionine, or catabolism through transsulfuration forming cystathionine (Eloranta *et al.*, 1990; Lieber and Packer 2002). Homocysteine can be remethylated through both folate-dependent and folate-independent mechanisms, both pathways contributing equally to the formation of methionine (Finkelstein and Martin 1984).

In hepatic tissue and possibly kidney, betaine derived from the oxidation of choline, serves as a source of methyl groups in the remethylation of homocysteine via the enzyme betaine-homocysteine *S*-methyltransferase (BHMT) (Finkelstein *et al.*, 1971). This folate-independent route results in methionine and DMG (Sunden *et al.*, 1997).

Alternatively, remethylation occurs with the donation of a methyl group by 5-CH₃THF through the enzymatic action of MS, a vitamin B₁₂-dependent enzyme (Finkelstein

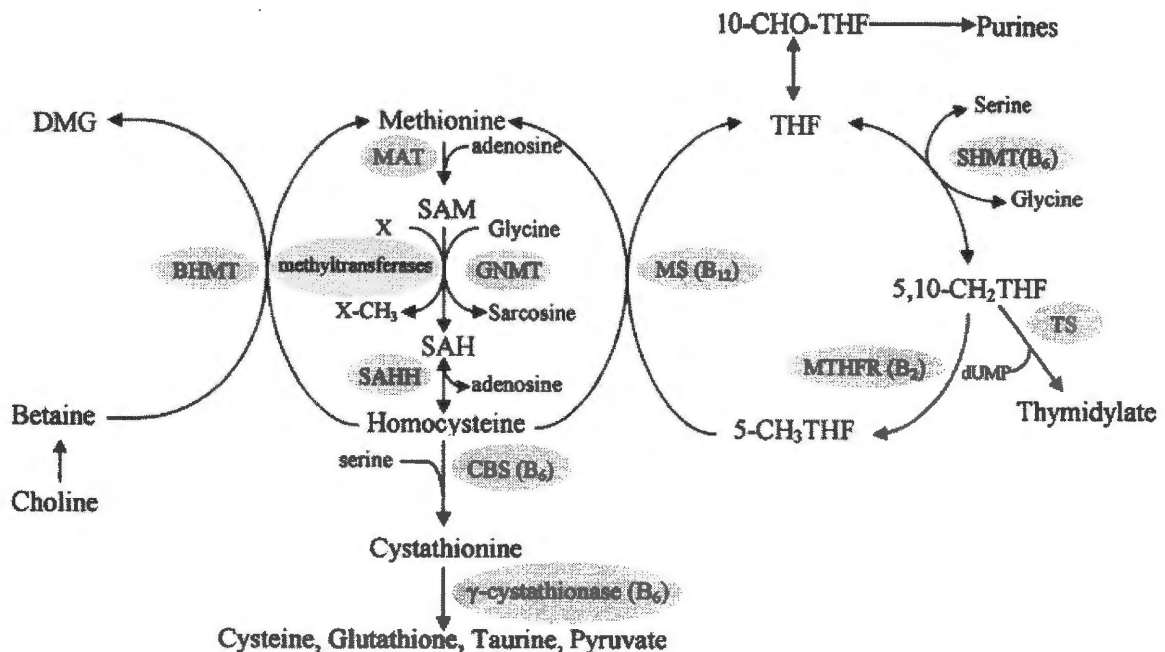


Figure 2.4. Folate, methyl group, and homocysteine metabolism

and Martin 1984). Upon donation of a methyl group in the remethylation of homocysteine, 5-CH₃THF is reduced to tetrahydrofolate (THF) (Wagner 1995). At this point THF can either be reversibly converted to 10-CHO-THF and donate its one-carbon units in the formation of purines, or combine with serine to form 5,10-CH₂THF and glycine. This reaction is reversible catalyzed by the enzymatic activity of serine hydroxymethyltransferase (SHMT), which is dependent on vitamin B₆ as a coenzyme (Schirch and Strong 1989). One-

carbon units from 5,10-CH₂THF, can then be donated to deoxyuridine monophosphate (dUMP) in formation of the DNA precursor, thymidylate via the catalytic activity of thymidylate synthase (TS) (Scott and Weir 1998) (**Figure 2.4**).

The folate compound 5,10-CH₂THF, is also used in the synthesis of 5-CH₃THF, which is irreversibly catalyzed by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR), a riboflavin-dependent enzyme. At any given time the concentration of free folate compounds in the cell is insignificant leading to a struggle for folate compounds between folate binding proteins, such as MTHFR and TS (Schirch and Stong 1989; Strong *et al.*, 1990; Suh *et al.*, 2001). The enzyme SHMT provides a regulatory mechanism for distribution of one-carbon units between thymidylate and methionine synthesis that when in excess favors thymidylate. This favoring is accomplished by binding and sequestering 5-CH₃THF which in turn prevents the remethylation of methionine (Herbig *et al.*, 2002).

Transsulfuration

Catabolism of the sulfur-containing amino acid homocysteine occurs through a group of reactions known as transsulfuration. Major enzymes involved in this pathway, cystathionine β-synthase (CBS) and γ-cystathionase, are vitamin B₆-dependent. The first committed step, converting homocysteine to cystathionine is catalyzed by CBS which utilizes serine in a condensation reaction (Kutzbach and Stokstad 1971; Krebs *et al.*, 1976). Finally, cystathionine is converted to the amino acid cysteine as well as other useful metabolites: glutathione, taurine, and pyruvate (Stipanuk *et al.*, 1992). The enzyme γ-cystathionase initiates this final step of the transsulfuration pathway (Stipanuk 1986). Transsulfuration does not occur ubiquitously, with some cells lacking part or the entire pathway (Finkelstein *et al.*, 1971).

Regulation of Methyl Group Metabolism

Allosteric Regulation

In order to make certain there is an adequate supply of methyl groups for SAM-dependent transmethylation reactions a number of regulatory mechanisms exist. The enzyme GNMT is subject to allosteric regulation based on the supply of methyl groups, in the form of SAM, to optimize the SAM to SAH ratio (Ogawa and Fujioka 1982). In the state of excess methyl groups through the diet or the folate-dependent one-carbon pool excess SAM binds to MTHFR, inhibiting enzyme activity (Jencks and Matthews 1987) (**Figure 2.5**). This inhibition reduces the synthesis of 5-CH₃THF and in turn alleviates the allosteric inhibition 5-CH₃THF inflicts upon GNMT resulting in the disposal of excess methyl groups. Conversely, in the state of methyl group deficiency, reduced circulating SAM concentrations results in increased activity of MTHFR elevating concentrations of 5-CH₃THF. Further, 5-CH₃THF allosterically inhibits GNMT thereby providing methyl groups for SAM-dependent transmethylation reactions (Wagner 1985).

In addition to regulating methyl group supply through the enzymes MTHFR and GNMT, there are a few other points of control. Specifically, SAM is a positive allosteric regulator of CBS, the enzyme that commits homocysteine to the transsulfuration pathway (Finkelstein *et al.*, 1975). Excess circulating SAM concentrations will activate CBS resulting in the normalization of methyl group supply by increasing catabolism. Secondly, excess dietary protein ensuing elevated SAM concentrations will restrict remethylation enzyme BHMT, again to bring methyl group supply to an optimal state (Finkelstein and Martin 1984). However if methionine is restricted, BHMT gene expression is enhanced, conserving methyl groups (Park *et al.*, 1997).

Hormonal Regulation

Specific hormones have been identified as having the ability to modulate methyl group metabolism. A diabetic state characterized by insufficient circulating insulin and

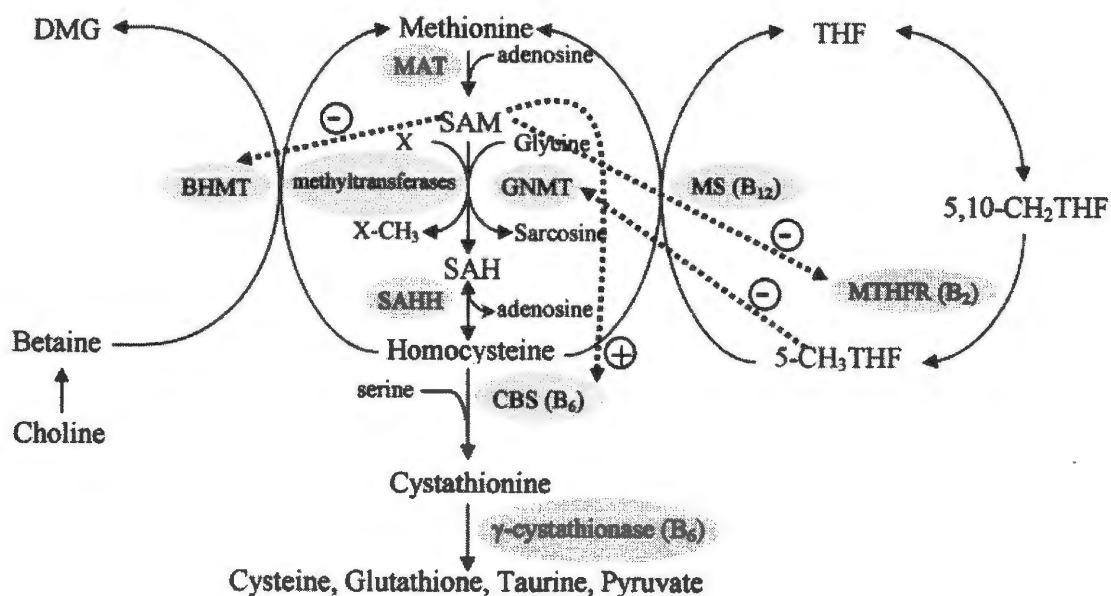


Figure 2.5. Regulation of methyl group metabolism

elevated counterregulatory hormones (e.g. glucagon, and glucocorticoids) has been indicated in directly altering enzymes involved in methyl group metabolism. In a study by Xue and Snoswell, a 65-fold induction in GNMT activity was reported in alloxan-induced diabetic sheep (1985). Similarly, in an alloxan-induced diabetic state or a starvation state in rats, resulted in a 2-fold induction in GNMT activity (Yeo and Wagner 1994).

Modifications in homocysteine concentrations are also evident in the diabetic state, but appear to be dependent on the status of the kidneys (Bostom and Culleton 1999;

Wolleson *et al.*, 1999). If renal function is sufficient, a diabetic condition results in a marked decrease in plasma homocysteine concentrations. However, if diabetes is coupled with renal dysfunction, the outcome is elevated plasma homocysteine levels (Poirier *et al.*, 2001).

Elevated homocysteine is an independent risk factor for vascular disease, which is considered to be the major cause of death in diabetics (Renold *et al.*, 1978). In a study by Jacobs *et al.*, researchers examined this effect in streptozotocin-induced rats and found plasma homocysteine concentrations were reduced by 40%; however treatment with insulin prevented the decrease in homocysteine, indicating insulin may be involved in the regulation (1998).

It seems that this decline in homocysteine manifests through the modification of transsulfuration enzymes, cystathionine β -synthase and γ -cystathionase. As presented in a later study by Jacobs *et al.*, a state of hyperglucagonemia in rats induced activity of CBS, γ -cystathionase, and increased CBS mRNA levels (2001). The study also revealed increased concentrations of SAM and SAH, which are allosteric activators of CBS, indicating an increase flux through the transsulfuration pathway (Jacobs *et al.*, 2001). Further, streptozotocin-induced diabetic rats in a study by Ratnam *et al.* revealed clearly elevated CBS mRNA which was reduced by insulin administration (2002).

Similarly, glucocorticoids have shown to modulate key enzymes in methyl group metabolism. In rat hepatoma cells, CBS protein abundance as well as CBS mRNA levels were increased following treatment with the synthetic glucocorticoid, triamcinolone. This effect occurred by stimulating gene expression at the transcriptional level, and the stimulatory effect was prevented by insulin treatment (Ratnam *et al.*, 2002). Moreover,

treatment with the glucocorticoid dexamethasone has shown to induce GNMT activity and protein abundance in rats and rat hepatoma cells (Rowling and Schalinske 2003).

Dietary Requirements

A sufficient supply of methyl groups through the diet, from choline and/or methionine, or as a result of the folate-dependent one-carbon pool is necessary for growth and development (Finkelstein and Martin 1986). Currently, only estimates exist for methionine requirements based on nitrogen balance studies, which tend to be grouped with the other sulfur-containing amino acid, cysteine. The estimates for methionine/cysteine requirements in mg/kg/day are classified by age as follows: 3-4 months, 158; 2-5 years, 27; 10-12 years, 22; greater than 12 years, 13 (Matthews 1999). It is definitely necessary to fulfill protein requirements, however excessive methionine can be toxic (Benevega and Steele 1984). The incorporation of choline into a low-protein diet at methyl loads at the same level or greater than methionine seems to reduce the adverse effects seen with methionine (Case *et al.*, 1976). Conversely, there is an adequate intake and UL stated for choline in the current DRIs. Adequate intake is given in $\mu\text{g}/\text{day}$ and classified by age as follows: 0-12 months, 125-150; 1-8 years, 200-250; 9-13 years, 375; males greater than 14 years, 550; females 14-18 years, 400; females greater than 18 years, 425. A slightly elevated choline recommendation exists for pregnant or lactating women at 450 and 500 $\mu\text{g}/\text{day}$, respectively (Food and Nutrition Board 2000).

Disruptions in Folate and Methyl Group Metabolism

Folate and methyl groups are directly or indirectly involved in cell function, division, and differentiation, thus necessary for essential biological processes. Therefore insufficient supply of folate, methyl groups, or related cofactors (vitamin B₆ and B₁₂) can result in a

multitude of pathologies as a result of disruptions in methyl group metabolism. In addition to nutrient deficiencies, treatment with retinoid compounds has shown to perturb methyl group metabolism. Lastly, individuals that possess genetic polymorphisms in key enzymes involved in these interrelated pathways may cause alterations and susceptibility to associated pathologies.

Neural tube defects

Folate and methyl group metabolism are critical components in fetus development given that one-carbon units are needed in the synthesis of DNA, protein, and phospholipids (Chiang *et al.*, 1996). Neural tube defects (NTDs) are characterized by malformation of the brain and/or spinal cord in the developing embryo, instigating incomplete central nervous system development (van der Put *et al.*, 2001). NTDs are one of the most common birth defects occurring in 1 in 1000 births in the United States (Center for Disease Control and Prevention 1996; AAP Committee on Genetics 1999).

The most common manifestations of NTDs are as congenital disorders, spina bifida and anencephaly, accounting for 50 and 40% of cases, respectively (Scott *et al.*, 1995). Spina bifida is distinguished by incomplete closure of the spinal cord (posterior end of the neural tube) causing physical disabilities in the child. Conversely, anencephaly results in incomplete closure of the skull (anterior end of the neural tube) and is incompatible with life (Green 2002). The etiology of NTDs is continually evolving but not yet clearly understood, with over 90% of NTD cases occurring from unknown causes (van der Put *et al.*, 2001). Identified origins of NTDs have been classified as both genetic and environmental with nutritional deficiency, specifically folate, being one of them (Lucock and Daskalakis 2000).

Folate status has long been examined for its role in NTDs, with earlier studies reporting associations between fetal malformations and folate deficiency (Hibbard 1964). Further interactions between NTDs and folate were found in study that reported low folate stores in women with pregnancies affected by NTDs, in turn suggesting periconceptional folate supplementation might protect against NTDs (Smithells *et al.*, 1976). Elucidating the specific mechanism by which folate supplementation prevents NTDs is ongoing; however data currently exists supporting the beneficial role of folate supplementation in reducing the occurrence of NTDs. In particular, it was shown that folic acid treatment alone was just as effective in preventing NTDs as a multivitamin with folate (Medical Research Council 1991). Moreover, a study involving 4,000 women reported 6 cases of NTDs in women receiving a placebo multi-mineral, as compared to no incidences of NTDs in the other group of women given a multivitamin containing folic acid (Cziezel and Dudas 1992). In addition, recent reports suggest an inverse relationship between methionine intake (Shoob *et al.*, 2001) and vitamin B₁₂ status (Schorah *et al.*, 1980; Kirke *et al.*, 1993), on the prevalence of NTDs. In response to the building evidence of the effectiveness of folic acid in the prevention of NTDs, the Center for Disease Control recommended any woman of child-bearing age consume at least 400 µg of folic acid per day (1991).

Hyperhomocysteinemia

Clearance of the non-protein-forming amino acid, homocysteine is dependent on remethylation and transsulfuration pathways and is possibly regulated nutritionally (Selhub 1999). SAM seems to be a key regulator in homocysteine metabolism since SAM allosterically inhibits MTHFR and BHMT and activates CBS. Folate as well vitamins B₆ and B₁₂ are also key factors in homocysteine metabolism. Deficiency in any of these nutrients

can result in the disruption in homocysteine metabolism, and in turn hyperhomocysteinemia (Green and Jacobsen 1995).

Hyperhomocysteinemia is positively associated with vascular diseases and has been recently added to the list of commonly known risk factors for cardiovascular disease (CVD) (Selhub and Miller 1992; Selhub *et al.*, 1995; Lee and Wang 1999). An elevation in plasma homocysteine concentrations by as little as 1 $\mu\text{mol/L}$ is associated with a 10% increase in the risk of CVD (Blom 1998). Severe hyperhomocysteinemia can result from inborn errors in enzymes necessary for clearance of homocysteine, specifically CBS, MTHFR, and MS (Clarke 1991; Selhub 1999). Folate deficiency has also been linked to elevated plasma homocysteine, with a 9.8-fold increase reported in folate-deficient rats as compared to folate-replete controls (Miller *et al.*, 1994). A reduction in plasma homocysteine concentrations were also evident following administration of folic acid promoting remethylation (Brattstrom *et al.*, 1988; Wilcken *et al.*, 1988; Mason and Miller 1992; Pancharuniti *et al.*, 1994). Therefore it is estimated that increasing folate intake by 200 μg per day will result in a reduction in homocysteine by 4 $\mu\text{mol/L}$ (Boushey *et al.*, 1995; van Oort *et al.*, 2003).

Research is ongoing as to whether homocysteine actually imposes atherogenic effects or if it just an indicator of another mechanism (Ueland *et al.*, 2000). Many studies do support the hypothesis that homocysteine is directly involved in vascular disease by causing degeneration of the vascular endothelium (Lucock and Daskalakis 2000). Particularly, a study using cultured endothelial cells has demonstrated cytotoxic effects following homocysteine treatment (Wall *et al.*, 1980). In addition an *in vivo* study revealed the rapid formation of plaques following injection of homocysteine thiolactone (McCully and

Ragsdale 1970). Additional mechanisms indicate homocysteine may induce vascular disease by causing platelet function abnormalities and endothelial dysfunction; however the exact mechanism is yet to be revealed.

Finally, elevated homocysteine is consistently associated with NTDs which again may be as a result of inborn errors in the enzymes (MTHFR and MS) involved in remethylation of homocysteine (van der Put *et al.*, 1995; Morrison *et al.*, 1997). Further an association has been identified between NTD births and increased cellular folate depletion. This was thought to be a result of impaired folate remethylation to THF the main intracellular form of folate (Lucock *et al.*, 1998). This further identifies the need to promote periconceptional vitamin supplementation, specifically folate.

Cancer

As explained previously, folate is directly and indirectly involved in the modification and synthesis of DNA. Therefore, folate deficiency has been identified as a factor contributing to cancer development and enhancement (Mason 1995). Mechanisms for this relationship have been proposed but not yet clearly defined (Bailey and Gregory 1999a). One hypothesis of this relationship is in the donation of one-carbon units from 5,10-CH₂THF in the synthesis of the DNA precursor thymidylate. Inadequate folate can lead to misincorporation of uracil into DNA due to lack of thymidylate. This misincorporation can lead to stress of the DNA repair mechanism and in turn cause DNA strand breaks and chromosome instability. However misincorporation of uracil and subsequent DNA damage seems to be reduced by folate supplementation (Blount *et al.*, 1997).

Another proposed mechanism for this relationship between folate and cancer is the role of folate in DNA methylation. DNA methylation has shown to regulate gene expression,

with lack of methylation typically leading to gene expression, and increased methylation resulting in gene suppression (Wagner 1995). In addition to methyl groups being provided by protein in the diet, folate indirectly plays a role in methylation of DNA by donating a methyl group to homocysteine in the generation of SAM. Therefore, it has been observed that rats fed a diet lacking folate, methionine, choline, and vitamin B₁₂, display decreased SAM concentrations and hepatic DNA methylation (Wainfan *et al.*, 1989). Global DNA hypomethylation has shown to precede cancer development, specifically due to hypomethylation of proto-oncogenes which increases transcription of associated mRNAs (Dizik *et al.*, 1991).

An additional hypothesis for the role of folate in cancer is in cytotoxic natural killer cells. Natural killer cells provide important immune protection against tumor cells (Trinchieri 1989). It was proposed that in folate deficiency these cells have reduced ability to destroy cancerous cells (Mason 1995). Further research suggests that natural killer cell-mediated cytotoxicity is dependent on the state of folate deficiency with severe folate deficiency in rats resulting in the reduced ability for cytotoxicity. However moderate folate deficiency, associated with increased risk of some cancers, did not affect cytotoxicity capabilities of natural killer cells (Kim *et al.*, 2002).

Accumulating evidence suggests that folate may play a role in development and progression of many cancers including: colorectum, lungs, pancreas, esophagus, stomach, cervix, breast, and leukemia (Kim 1999). The most evidence of the relationship between folate and cancer development has been exposed in colorectal cancer (Prinz-Langenohl *et al.*, 2001; Kim 2003). Several retrospective studies have suggested a decreased risk of colorectal

neoplasms in subjects with the highest folate intake; whereas folate deficiency increases the risk of colorectal cancer (Bailey *et al.*, 2003; Giovannucici 2002).

Megaloblastic Anemia

Disturbed DNA synthesis as a result of folate and/or B₁₂ deficiency can result in the megaloblastic anemia. This form of anemia manifests in large, immature, abnormal, red blood cell precursors in the bone marrow and peripheral blood (Kasdan 2000). The role folate and vitamin B₁₂ play in this pathology is in the pathway converting 5-CH₃THF to THF through the activity of MS (Savage and Lindenbaum 1995). In the case that there is vitamin B₁₂ deficiency, MS activity is reduced essentially trapping methyl groups in the form 5-CH₃THF. This “methyl trap” decreases the availability of folate coenzymes for purine and pyrimidine biosynthesis, which are needed for red blood cell division and differentiation (Shane and Stokstad 1985).

This somewhat pseudo folate deficiency that occurs can result in a reduction of SAM and in turn methyl groups for transmethylation reactions including myelin basic protein. A decrease in methyl groups for synthesis of myelin basic protein can cause demyelination and in turn a state of neuropathy (Scott 1999). Therefore, it is crucial that individuals suffering from megaloblastic anemia not only supplement folate, but vitamin B₁₂ to prevent associated neuropathy. However, steps should be taken to discover the root of the problem because supplementing folate can mask vitamin B₁₂ deficiency and exacerbate associated neurological problems (Bottiglieri *et al.*, 1995).

Neurological/Neuropsychiatric Disorders

Neurological disorders associated with folate and/or B₁₂ deficiency are not quite as well understood as the hematological disorders. Vitamin B₁₂ and folate deficiencies have

been associated with neurological and neuropsychiatric disorders (Bottiglieri 1996). A hypothesis for the role of these nutrients is again through the reaction catalyzed by MS. MS, which utilizes both vitamin B₁₂ and folate, in the formation of methionine and in turn SAM. SAM provides methyl groups in the synthesis of proteins, phospholipids and neurotransmitters such as serotonin, norepinephrine, and dopamine. Specifically reduced serotonin levels and activity have been reported in the brain of folate-deficient rats and humans (Hyland *et al.*, 1988; Botez *et al.*, 1979). It is interesting to note that 5-CH₃THF is transported across the blood brain barrier, and the concentration in the cerebrospinal fluid is three times greater than in serum (Bottiglieri *et al.*, 1995). This further indicates the importance of the MS-catalyzed pathway.

Several studies have reported a high incidence of folate deficiency accompanying depression (Carney *et al.*, 1990; Morris *et al.*, 2003). Studies have also been conducted to determine the efficacy of folate and SAM supplementation in these affective disorders, whereas supplementation seemingly promotes antidepressant activity (Freidal *et al.*, 1989; Godfrey *et al.*, 1990). However it is not clear if folate deficiency actually plays a role in the development of depressive illnesses or if it is secondary to poor appetite.

Recent studies have also linked the rise in homocysteine, resulting from folate deficiency and reduced enzyme activity, to two major neurological disorders: Alzheimer's disease and Parkinson's disease (Mattson and Shea 2003). Typically serum homocysteine levels are elevated in folate- or vitamin B₁₂-deficient patients (Allen *et al.*, 1993). There are several suspected mechanisms for the role of folate and homocysteine in neuronal damage. One of these mechanisms is folate deficiency is thought to induce DNA damage by reducing homocysteine remethylation to SAM for transmethylation reactions (Selhub and Miller

1992). Another possible mechanism is the accumulation of SAH. Elevated homocysteine creates a favorable condition for the hydrolysis of homocysteine to SAH by SAHH. SAH accumulation will inhibit SAM-dependent transmethylation reactions, and in turn induced DNA damage and cell apoptosis (Kruman *et al.*, 2000). In addition, it has been shown that neurons cultured with homocysteine deplete their ATP reserves in attempts to repair damaged DNA. ATP depletion is thought to be a key factor in the degeneration of neurons in Alzheimer's and Parkinson's diseases (Kruman *et al.*, 2000).

Genetic Profile

Genetic polymorphisms are among the factors that can perturb folate and methyl group metabolism resulting in associated pathologies. These genetic differences are in key enzymes in one-carbon metabolism and typically result in reduced activity. Several of these polymorphisms have been identified as well as the possible effects of their existence.

MTHFR polymorphism

The enzyme MTHFR catalyzes the irreversible conversion of 5,10-CH₂THF to 5-CH₃THF, which donates its methyl group in the remethylation of homocysteine. The most common mutation of this enzyme is autosomal recessive, occurring at base pair 677, and resulting in a C→T substitution (C677T) causing valine to substitute for alanine in the enzyme (Frosst *et al.*, 1995). The frequency of this mutation varies depending on race and ethnic background, but it is estimated to occur at rate of 12% in Caucasian and Asian populations homozygous for the trait, and up to 50% for those who are heterozygous for the trait (Brattstrom *et al.*, 1998).

Individuals that are homozygous for this mutation exhibit reduced activity and stability of MTHFR. Studies indicate that persons with the homozygous C677T mutation

have reduced plasma folate concentrations and elevated plasma homocysteine concentrations (Brattstrom *et al.*, 1998). Modest elevations in homocysteine is an independent risk factor for vascular disease and NTDs; however studies are conflicting as to whether the homozygous C677T mutation is itself a risk factor in vascular disease and NTDs (Bailey and Gregory 1999b). The relationship between cancer and the homozygous C677T mutation seems to be dependent on folate status. A decreased risk for colorectal cancer was found in men with normal plasma folate concentrations and the MTHFR mutation by increasing the availability of one-carbon units for nucleotide synthesis. Conversely, the men with low folate status seemed to counteract the protective effect of the mutation (Ma *et al.*, 1997).

MS polymorphism

The enzyme MS has also been identified in the list of mutations associated with one-carbon metabolism. The most common MS polymorphism is located at base pair 2756 causing an A→G (A2756G) substitution and resulting in glycine replacing aspartic acid in the functional enzyme (Chen *et al.*, 1997). It is not yet clear whether this mutation changes activity or abundance of the enzyme; however most research indicates there is no association between this mutation and homocysteine concentrations (Hyndman *et al.*, 2000; Klerk *et al.*, 2003). Most studies also indicate that there is no association between the homozygous mutation for MS and the risk for vascular disease (Ma *et al.*, 1999; Morita *et al.*, 1999; Tsai *et al.*, 1999). Although a study by Kerk *et al.* did find that subjects homozygous for the MS mutation exhibited a four-fold increase in the risk of coronary heart disease as compared to individuals with the wild-type or heterozygous genotypes (2003).

CBS enzyme deficiency

The transsulfuration pathway beginning, with the CBS activity, plays a crucial part in the removal of homocysteine when the supply of methyl groups is in excess (Finkelstein and Martin 1984). Therefore, when there is a deficiency in CBS activity both hyperhomocysteinemia and homocystinuria develop, and manifest in a number of pathologies (Selhub 1999). CBS enzyme deficiency is an autosomal recessive trait and the most common inborn error of methionine metabolism occurring in approximately 1 in 200,000 individuals worldwide (Selhub and Miller 1992). Specifically, osteoporosis, scoliosis, arteriosclerosis, thromboembolism, convulsions, and psychiatric disturbances are some problems that can develop from this enzyme deficiency (Kluijtmans *et al.*, 1999), and as many as 50% of those who go untreated, develop mental retardation (Yaghmai *et al.*, 2002). The exact mechanism by which CBS enzyme deficiency is associated with diseases is not yet known.

Greater than 60 mutations have been identified in the CBS gene (Kraus 1998). The most severe mutations occur at base pair 833 resulting in a T→C (T833C) substitution and at base pair 919 resulting in a G→A substitution (G919A). These mutations are fairly rare affecting less than 1% of the general population (Kluijtmans *et al.*, 1996). The most common mutation is caused by a 68 base pair insertion between nucleotides 844 and 845 (844ins68). This mutation occurs in the heterozygous form in approximately 12 % of the U.S. population (Tsai *et al.*, 2000).

CBS is a vitamin B₆-dependent enzyme, therefore some patients with CBS deficiency respond to vitamin B₆ supplementation resulting in lower homocysteine concentrations. It is estimated that approximately 50% of those with CBS enzyme deficiency respond to high

doses of vitamin B₆, evident by lowered homocysteine concentrations (Mudd *et al.*, 1985). However, many individuals do not respond to vitamin B₆ supplementation and therefore must rely on a methionine-restricted diet. Vitamins B₁₂, B₆, and folate may further reduce homocysteine concentration in these resistant individuals (Wilcken and Wilcken 1997). Betaine supplementation has also been tested in CBS deficient patients and has been found to consistently lower plasma homocysteine concentrations. The only concern is the elevated methionine levels that result, however most patients seem to remain below 1500 µmol/L, a level that has not been associated with adverse effects (Wilcken *et al.*, 1983; Wilcken and Wilcken 1997).

Retinoids

Factors both nutritionally and hormonally have been identified as having the ability to alter methyl group metabolism by targeting key enzymes involved. Specifically the vitamin A derivatives, all-*trans*-retinoic acid (RA), and 13-*cis*-retinoic acid (CRA) are among these factors. More importantly these alterations may affect those who utilize these derivatives therapeutically. CRA (Isotretinoin/Accutane®) is used in dermatology and currently taken orally to treat cystic acne that is unresponsive to topical treatment; whereas RA (Vesanoid®) is used in oncology to treat promyelocytic leukemia (Ohno *et al.*, 2003; Peck *et al.*, 1979). It is also interesting to note that the number of CRA prescriptions dispensed to treat cystic acne have increase 250% in the last decade (Wysowski *et al.*, 2002).

There are a number of side effects that can result from the therapeutic use of retinoids. Aside from CRA being a known teratogen, the most common side effect of retinoid administration is dyslipidemia resulting in elevated plasma lipid concentrations (Bershad *et al.*, 1985). Approximately 90% of the vitamin A in the body is stored in the

liver, so excessive vitamin A can lead to liver abnormalities (Roenigk 1988). Administration of CRA in rats has also revealed accumulation of hepatic lipids in addition to elevated serum triglycerides (Schalinske and Steele 1993). Retinoid administration has shown to effect one-carbon metabolism by increasing methionine catabolism as well as inducing hypomethylation of DNA by activating GNMT in rats (Fell and Steele 1986; Schalinske and Steele 1991; Rowling *et al.*, 2002). An additional study also found elevated homocysteine concentrations in humans treated with isotretinoin, linking this finding to either CBS inhibition or liver dysfunction (Schulpis *et al.*, 2001)

CRA and RA have shown to induce GNMT activity and protein abundance *in vivo* (Rowling and Schalinske 2001; McMullen *et al.*, 2002; Ozias and Schalinske 2003) as well as induction of GNMT by RA *in vitro* (Rowling and Schalinske 2003). Induction of GNMT results in a loss of methyl groups for subsequent SAM-dependent transmethylation reactions. This effect seems to be dose-dependent with as little as 5 $\mu\text{mol/kg}$ body weight and a single dose causing significant induction and a maximal effect at 30 $\mu\text{mol/kg}$ body weight and four days of treatment (Ozias and Schalinske 2003). Activation of GNMT by retinoids also exhibited tissue and gender specificity. McMullen *et al.* reported male rats were more sensitive to retinoid-mediated activation of GNMT than female rats (2002). Retinoid administration resulted in elevated hepatic GNMT protein and abundance, but did not affect renal or pancreatic GNMT (McMullen *et al.*, 2002).

RA is known to induce a gluconeogenic state which in turn alters methyl group metabolism through the induction of key gluconeogenic enzymes including phosphoenolpyruvate carboxykinase, the rate-limiting step of gluconeogenesis (Pan *et al.*,

1990; Shin and McGrane 1997). Interestingly, GNMT is located primarily in gluconeogenic tissues implying a possible role in hormonal regulation. These modifications of GNMT activity by RA are thought to occur transcriptionally, however there is currently no report of a retinoic acid response element on the promoter region of GNMT (Pan, *et al.*, 1990).

Phosphorylation of GNMT has been shown to modulate activity of the enzyme. Wagner *et al.* found *in vitro* that phosphorylation, by cAMP-dependent protein kinase, of GNMT resulted in a 2-fold increase in the activity of the enzyme; however if 5-CH₃THF was first bound to GNMT, phosphorylation was inhibited as well as enzyme activity (1989). Further research needs to be conducted to exactly determine the mechanism by which retinoids alter methyl group metabolism. These factors that affect methyl group metabolism prompted the study in the following chapter. The study was conducted to determine how both a diabetic state and/or retinoid treatment alter methyl group metabolism.

CHAPTER 3 - MODULATION OF METHYL GROUP METABOLISM BY STREPTOZOTOCIN-INDUCED DIABETES AND ALL-*trans*- RETINOIC ACID¹

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Abstract

The hepatic enzyme glycine *N*-methyltransferase (GNMT) plays a major role in the control of methyl group and homocysteine metabolism. Because disruption of these vital pathways is associated with numerous pathologies, understanding GNMT control is important for evaluating methyl group regulation. Recently, gluconeogenic conditions have been shown to modulate homocysteine metabolism and treatment with glucocorticoids and/or all-*trans*-retinoic acid (RA) induced active GNMT protein, thereby leading to methyl group loss. This study was conducted to determine the effect of diabetes, alone and in combination with RA, on GNMT regulation. Both diabetes and RA increased GNMT activity 87% and 148% respectively. Moreover, the induction of GNMT activity by diabetes and RA was reflected in its abundance. Cell culture studies demonstrated that pretreatment with insulin prevented GNMT induction by both RA and dexamethasone. There was a significant decline in homocysteine concentrations in diabetic rats, owing in part to a 38% increase in the abundance of the transsulfuration enzyme cystathionine β -synthase (CBS); treatment of diabetic rats with RA prevented CBS induction. A diabetic state also increased the activity of the folate-independent homocysteine remethylation enzyme betaine-homocysteine S-

methyltransferase (BHMT), whereas the activity of the folate-dependent enzyme methionine synthase (MS) was diminished 52%. In contrast, RA treatment attenuated the streptozotocin-mediated increase in BHMT, whereas MS activity remained diminished. These results indicate that both a diabetic condition and RA treatment have marked effects on the metabolism of methyl groups and homocysteine, a finding that may have significant implications for diabetics and their potential sensitivity to retinoids.

Introduction

Methyl group and folate-dependent one-carbon metabolism are interrelated pathways that provide for the subsequent transmethylation of various molecules using S-adenosylmethionine (SAM) ⁶. SAM, resulting from the adenylation of methionine, is the primary methyl donor in the body (Wagner 1995) (**Figure 3.1**). An adequate supply of methyl groups, via the diet or the folate-dependent one-carbon pool, and regulation of these pathways is essential in preventing associated pathologies such as cancer, vascular disease, and neural tube defects (Ross 2003; Kang *et al.*, 1992; Scott *et al.*, 1990). Glycine N-methyltransferase (GNMT) is an abundant, tissue-specific protein that plays a key role in the regulation of hepatic methyl group metabolism by the enzymatic conversion of glycine and SAM to S-adenosylhomocysteine (SAH) and sarcosine (Wagner 1995; Kerr 1972; Yeo and Wagner 1994). GNMT functions to optimize the SAM/SAH ratio, an indicator of transmethylation potential, because SAH is a potent inhibitor of most SAM-dependent methyltransferases (Kerr 1972; Cantoni and Chiang 1980). Following hydrolysis of SAH, the resulting homocysteine can undergo remethylation to methionine, or be irreversibly catabolized by the transsulfuration pathway. Folate-dependent remethylation occurs with the

donation of a methyl group by 5-methyltetrahydrofolate (5-CH₃-THF) through the action of B₁₂-dependent methionine synthase (MS) (Shane and Stokstad 1985). In hepatic tissue, betaine derived from the oxidation of choline can also serve as a folate-independent source of methyl groups for homocysteine remethylation via the enzyme betaine-homocysteine S-methyltransferase (BHMT). Transsulfuration to cysteine occurs through the activity of two vitamin B₆-dependent enzymes. Initially, homocysteine is converted to cystathionine by the activity of cystathionine β-synthase (CBS), and finally, γ-cystathionase converts cystathionine to the amino acid cysteine (Jacobs *et al.*, 2001).

The regulation of hepatic GNMT represents an important mechanism for controlling both the folate-dependent supply of methyl groups as well as their utilization in SAM-dependent transmethylation reactions. GNMT activity is regulated in response to changes in methyl group status as the result of allosteric inhibition of 5,10-methylenetetrahydrofolate reductase (MTHFR) by SAM (Jencks and Matthews 1987; Kutzback and Stokstad 1967) and inhibition of GNMT by 5-CH₃-THF (Wagner *et al.*, 1985; Wagner *et al.*, 1989), the enzymatic product of MTHFR. This allows methyl groups to be conserved under conditions of deficiency by decreasing GNMT activity, whereas elevations in its activity function to dispose of excess methyl groups. In addition to allosteric control, phosphorylation of GNMT represents another posttranslational mechanism to increase the activity of GNMT and regulate methyl group metabolism (Wagner *et al.*, 1989).

Recent studies have identified both nutritional and hormonal factors that alter methyl group metabolism by targeting key enzymes involved. All-*trans*-retinoic acid (RA) has been shown to induce hepatic GNMT activity and protein abundance, resulting in the loss of methyl groups for subsequent transmethylation reactions (McMullen *et al.*, 2002; Ozias

and Schalinske 2003; Rowling *et al.*, 2002; Rowling and Schalinske 2001). Moreover, dexamethasone (DEX) was just as effective as RA in the induction of GNMT activity in rat liver and hepatoma cells, and the coadministration of both DEX and RA induced GNMT in an additive fashion (Rowling and Schalinske). A diabetic state characterized by insufficient circulating concentrations of insulin and elevated levels of the counter-regulatory hormones, glucagon and glucocorticoids, has also been reported to alter enzymes involved in methyl group metabolism. An elevation in the activity of GNMT has been reported in alloxan-induced diabetic sheep and rats (Yeo and Wagner 1994; Xue and Snoswell 1985); however, little is known about the mechanistic basis for this increase. Brosnan and coworkers have shown that under diabetic conditions, the catabolism of homocysteine was enhanced by transcriptional regulation of CBS and these changes were prevented by treatment with insulin (Jacobs *et al.*, 1998; Jacobs *et al.*, 2001; Ratnam *et al.*, 2002).

It has become overtly evident that both treatment with RA and a diabetic state independently cause aberrant modifications in methyl group metabolism. Although we have shown that DEX can significantly alter methyl group and homocysteine metabolism (Rowling and Schalinske 2003), the focus in this study was to evaluate these pathways in a diabetic rat model, alone and in combination with RA, as well as determine the effect of insulin on preventing these alterations.

Experimental Procedures

Chemicals

Reagents were obtained as follows: S-adenosyl-L-[methyl-³H]methionine, Perkin Elmer Life Sciences (Boston, MA); chemiluminescence Western blotting detection reagents, Amersham Pharmacia (Piscataway, NJ); S-adenosyl-L-methionine and streptozotocin, Sigma

Chemical (St. Louis, MO); protease inhibitors, and RA, Calbiochem (Lamoille, CA); goat anti-mouse IgG horseradish peroxidase and goat anti-rabbit IgG horseradish peroxidase, Southern Biotechnology (Birmingham, AL). GNMT and CBS antibodies were kindly provided by Yi-Ming Chen, National Yang-Ming University, Taipei, Taiwan (Liu *et al.*, 2003) and Jan Kraus, University of Colorado Health Sciences Center, Denver, CO, respectively. All other chemicals were of analytical grade.

Animals

All animal experiments were approved by and conducted in accordance with Iowa State University Laboratory Animal Resources Guidelines. Male Sprague-Dawley (Harlan Sprague Dawley, Indianapolis, IN) rats (125-149g) were housed in individual plastic cages in a room with a 12-h light:dark cycle, and allowed free access to food and water. Rats were randomly assigned to treatment groups (6 rats per group) and acclimated to the control diet (Rowling and Schalinske 2003) for 5 d. Following the acclimation period, rats received a single intraperitoneal injection of either streptozotocin (STZ; 60 mg/kg body weight) in the vehicle (10 mM citrate buffer, pH 4.5) or the vehicle alone. The following day animals received a daily oral dose of RA (30 μ mol/kg body weight) or vehicle (corn oil) alone. Following a 5-d treatment period with RA, rats were anesthetized and whole blood samples were collected via cardiac puncture. An aliquot of whole blood was used to assess blood glucose concentrations using a commercial kit (Sigma Chemical, St. Louis, MO), whereas the remaining blood was centrifuged at $4,000 \times g$ for 6 min and the resulting plasma fraction was stored at -20°C for subsequent analysis of homocysteine concentrations. Liver samples were removed and homogenized in 4 volumes of ice-cold phosphate buffered (10 mM, pH 7.0) sucrose (0.25 M) containing 1 mM EDTA, 1 mM sodium azide, and 0.1 mM

phenylmethylsulfonyl fluoride (PMSF). Following centrifugation at $20,000 \times g$ for 30 min, supernatants were stored at -70°C with 1mM β -mercaptoethanol for subsequent enzymatic and protein abundance measurements. Total soluble protein concentrations were determined based on the method of Bradford (Bradford 1976) using a commercial kit (Coomassie Plus, Pierce Chemical, Rockford, IL) and bovine serum albumin as a standard. Additional liver samples were homogenized in 2 volumes of 0.4 M perchloric acid for the determination of SAM and SAH.

Cell Culture

Rat pancreatic AR42J and hepatoma H4IIE cells were obtained from the American Type Culture Collection (Manassas, VA). All cells were grown in 150-cm² flasks in a humidified incubator with 5% CO₂ and a temperature of 37°C until they were 70-75% confluent. H4IIE cells were cultured in minimum essential medium containing 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (0.1 mg/mL). AR42J cells were grown in F-12K nutrient mixture containing 20% fetal bovine serum as well as penicillin and streptomycin. Cell lines received fresh media prior to the initiation of any treatments. H4IIE and AR42J cells were treated with either a vehicle of 0.01% dimethyl sulfoxide (Me₂SO, control), 10 μM RA, 0.1 μM DEX, or both RA and DEX as described previously (Rowling and Schalinske 2003). A parallel group of AR42J and H4IIE cells were preincubated with 100 nM insulin for 24 h prior to the addition of RA and/or DEX. Following a 72-h incubation period, cells were detached using 0.25% trypsin/ 1mM EDTA, washed with Hank's balanced salt solution, and lysed on ice in a buffer containing 10 mM HEPES (pH 7.4), 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 50 mM β -glycerophosphate, 5 mM EDTA, 1 mM sodium orthovanadate, 2 mM benzamidine, 100 $\mu\text{g}/\text{mL}$ leupeptin and

pepstatin, 250 µg/mL soybean trypsin inhibitor, 0.2 mM PMSF, 24 µg/mL *p*-nitroguanidinobenzoate, and 0.5% Nonidet P-40. Following centrifugation at 16,000 × *g* for 8 min, supernatants were stored at -70°C for analysis of GNMT protein abundance.

GNMT Activity Analysis

The enzymatic activity of GNMT was determined using the method described by Cook and Wagner (Cook and Wagner 1984) with minor modifications, and performed in triplicate. The assay mixture (100 µL) consisted of 0.2 M Tris buffer (pH 9.0), 2 mM glycine, 5 mM dithiothreitol, 0.2 mM S-adenosyl-L-[methyl-³H]methionine, and was initiated with 250 µg protein followed by incubation at 25°C for 30 min. Trichloroacetic acid (10%) was added to terminate the reaction followed by the addition of activated charcoal and centrifugation (14,000 × *g*) to remove radiolabeled SAM. Aliquots of the supernatants were removed for liquid scintillation counting.

Determination of GNMT and CBS Protein Abundance

GNMT protein abundance was determined using immunoblotting techniques as previously described (Rowling and Schalinske 2003). A 10-20% gradient SDS-polyacrylamide gel was used to quantify the 32-kDa monomer subunit of GNMT. Following separation, proteins were transferred to a nitrocellulose membrane and incubated overnight at 4°C with a 1:4,000 dilution of the monoclonal GNMT antibody (Liu *et al.*, 2003). The membrane was then incubated with goat anti-mouse IgG horseradish peroxidase secondary antibody for 1 h at room temperature. CBS protein abundance was analyzed using a similar method as described above to separate the 63-kDa CBS subunit. A 1:20,000 dilution of the polyclonal CBS antibody was used, followed by incubation with a goat anti-rabbit IgG horseradish peroxidase secondary antibody. GNMT and CBS protein abundance were

detected with chemiluminescence and exposed to Kodak X-Omat AR film. Densitometric analysis was performed using SigmaGel software (SPSS, Chicago, IL). For both GNMT and CBS, three samples from each treatment group were randomly chosen for analysis on a single immunoblot.

Determination of Hepatic SAM and SAH Concentrations

Perchloric acid-homogenates were centrifuged at $9,000 \times g$ for 10 min and the resulting supernatants were neutralized and applied to a C₁₈ SepPak cartridge (Waters, Milford, MA) to obtain SAM and SAH (Fell *et al.*, 1985; Schalinske and Steele 1996). SAM and SAH were separated and quantified by reversed-phase HPLC and UV detection (254 nm) using a mobile phase containing 30% methanol in 5 mM octane sulfonic acid (pH 4.0) operated isocratically at 1.2 mL/min.

Analysis of BHMT and MS Activity

BHMT activity was measured as previously described by Garrow (Garrow 1996). The standard BHMT assay contained 5 mM DL-homocysteine, 2 mM betaine (0.1 μ Ci), and 40 μ g total protein. The activity of MS was determined as previously described (Keating *et al.*, 1985). The assay reaction mixture (200 μ L) containing sodium phosphate buffer (105 mM, pH 7.5), cyanocobalamin (0.12 μ M), dithiothreitol (36 mM), SAM (0.3 mM), β -mercaptoethanol (8.4 mM), homocysteine (8.25 mM), and [*methyl*-¹⁴C]-THF (0.63 mM, 0.17 μ Ci/ μ mol) was added to liver supernatants, each sample containing 600 μ g of protein, and incubated for 1 h at 37°C. Ice-cold water was added to terminate the reaction and the assay mixture was immediately applied to an AG 1-X8 (chloride form) resin columns. Effluent fractions (3 mL deionized water) were collected for subsequent liquid scintillation counting.

For both the BHMT and MS assay, homocysteine was prepared fresh daily from a thiolactone derivative.

Plasma Homocysteine Analysis

Total plasma homocysteine concentrations were determined using HPLC and fluorescence detection (Ubbink *et al.*, 1991). For derivatization, 10% tributylphosphine in dimethylformamide was added to 300 μ L plasma samples and subsequently incubated at 4°C for 30 min. The reaction was terminated with ice-cold trichloroacetic acid containing 1 mM EDTA. Following centrifugation at 1,000 \times g for 5 min, supernatants were added to a solution containing borate buffer (0.125 M, pH 9.5), sodium hydroxide (1.55 M), and 4-fluoro-7-sulfobenzofurazan (ammonium salt, 0.1%). *N*-acetylcysteine (1 mM) was added to the plasma samples prior to derivatization as an internal standard. Samples were injected onto a μ Bondapak C₁₈ Radial-Pak column (Waters, Milford, MA) equilibrated in a mobile phase consisting of 4% acetonitrile in 0.1 M potassium phosphate buffer (pH 2.1).

Statistical Analysis

SigmaStat software (SPSS, Chicago, IL) was used for all statistical analyses. The mean values of each treatment group were subjected to a two-way ANOVA. When the ANOVA was significant ($p < 0.05$), means were compared using Fisher's least significant difference procedure.

Results

Rats Treated with STZ Resulted in Elevated Blood Glucose and Significantly Less Weight Gain Regardless of RA Treatment

Cumulative weight gain for diabetic rats was 23 and 36% of respective non-diabetic values, whereas RA treatment was without effect (**Table 3.1**). STZ clearly induced a

diabetic state in rats as blood glucose concentrations were elevated 3.7-fold compared to control values. Rats treated with RA had no significant effect on circulating levels of glucose compared to control values, and RA attenuated the hyperglycemia exhibited by diabetic rats.

Both Diabetes and RA-treatment Induced Active GNMT

STZ-treated rats exhibited a 1.9-fold increase in hepatic GNMT activity compared to the control values (**Figure 3.2A**). RA increased GNMT activity 2.5-fold compared to control rats and was significantly greater than the degree of induction in STZ-treated rats. Hepatic GNMT activity was highest in the rats receiving both STZ and RA (2.6-fold), although this increase was not significantly different from RA treatment alone. The changes in GNMT activity in diabetic and RA-treated rats were also reflected in the abundance of the protein, where a 5.3- and 4.8-fold increase was observed, respectively (**Figure 3.2B**). Moreover, co-administration of STZ and RA significantly induced GNMT protein abundance (6.8-fold) to a greater extent than either treatment alone. Thus, both RA treatment and a diabetic condition modulate the expression of active GNMT in rat liver.

Induction of GNMT by RA and/or DEX was Prevented by Insulin Treatment

As reported previously (Rowling and Schalinske 2003), both RA and DEX alone or in co-administration resulted in induction of the GNMT protein in H4IIE rat hepatoma cells (**Figure 3.3**). Incubating cells with insulin for 24 h prior to treatment with RA and/ or DEX prevented GNMT induction. Similarly, DEX-mediated induction of GNMT in AR42J cells was also prevented by insulin; however, RA was not an effective signal to induce GNMT in these cells.

STZ-treatment Reduced Plasma Homocysteine Concentrations and Induced CBS Abundance

Plasma homocysteine concentrations were significantly reduced ~48% in both groups of STZ-treated rats, regardless of RA administration (**Figure 3.4**). Although we have previously found that RA alone was effective at lowering circulating homocysteine levels (Ozias and Schalinske 2003), the 26% decrease observed under these experimental conditions did not reach statistical significance. The hypohomocysteinemia exhibited by diabetic rats appears to be due, in part, to a 38% increase in the abundance of CBS, the initial enzyme in the irreversible catabolism of homocysteine (**Figure 3.5**). However, CBS activity is regulated allosterically and thus, flux studies conducted in vivo would be required to evaluate the transsulfuration pathway. Interestingly, RA treatment of diabetic rats prevented CBS induction (**Figure 3.5**), even though plasma homocysteine concentrations remained diminished (**Figure 3.4**).

STZ- and RA-treatments Differentially Stimulate Homocysteine Remethylation Enzymes

In addition to transsulfuration, the reduced plasma homocysteine levels also appear to reflect changes in the activity of enzymes involved in homocysteine remethylation. BHMT activity was significantly increased 3.3-fold in diabetic rats, whereas the activity of MS was reduced 52% (**Table 3.2**). RA did not significantly alter either enzyme, although there was a trend ($p = 0.061$) for increased MS activity, similar to our previous observations (Ozias and Schalinske 2003). RA was effective at reducing the activity of BHMT in diabetic rats, but did not prevent the STZ-mediated decrease in MS activity.

STZ-mediated Elevations in the Hepatic Concentrations of SAM and SAH were Prevented by RA Treatment

A diabetic condition resulted in a significant increase in the hepatic concentrations of SAM (169%) and SAH (90%). Although RA treatment did not significantly alter SAM and SAH concentrations in non-diabetic rats, it did prevent their accumulation in diabetic animals (**Table 3.3**). Because SAM and SAH are allosteric regulators of a number of enzymes involved in folate, methyl group, and homocysteine metabolism, it is difficult to determine the collective metabolic effect of these changes in diabetic rats in the absence of flux measurements using isotopic tracers.

Discussion

Methyl groups, homocysteine, and the folate-dependent one-carbon pool represent interrelated metabolic processes that are important in health and disease. Because GNMT is a vital regulatory protein that functions to control the supply and utilization of methyl groups, it is paramount to understand how nutritional and/ or hormonal factors modulate its expression. Our earlier work demonstrated that RA and DEX were independent signals to induce active GNMT in rat liver and in cultured hepatoma cells (Rowling and Schalinske 2003). Here, we have extended these findings by demonstrating that a diabetic condition, mediated by treatment with STZ, also has a profound impact on the activity of hepatic GNMT. Previous rat studies using alloxan-induced diabetes reported similar increases (85%) in GNMT activity (Yeo and Wagner 1994), whereas treatment with glucagon were less pronounced (23%) (Jacobs *et al.*, 2001); however, the mechanistic basis for this increase in GNMT activity has not been thoroughly explored. A novel aspect of our studies demonstrates that for both diabetes and RA, the abundance of GNMT protein was markedly

elevated, indicating that GNMT was regulated by transcriptional and/or translational mechanisms, or possibly at the level of degradation of the protein. It is not known if regulation of GNMT expression in a diabetic state is the result of an increase in glucocorticoid levels or a lack of insulin. The cell culture studies shown here clearly demonstrate that pretreatment with insulin prevented GNMT induction by both STZ and RA. It will be important in future studies to more fully characterize this diabetic condition with alterations in methyl group metabolism. Interestingly, the responsiveness of GNMT expression to RA and DEX appears to be cell-type specific. We have found in other studies that DEX was a more universal signal than RA for GNMT induction across a number of tissues and cell lines, including those that do not express detectable GNMT, such as the human hepatoma cell line HepG2⁷.

It is not clear how RA, DEX, and/or a diabetic state alter the expression of GNMT. Previous studies on the regulation of GNMT activity have found it to be at the posttranslational level by phosphorylation and allosteric inhibition by 5-CH₃-THF (Wagner *et al.*, 1985; Wagner *et al.*, 1989; Balaghi *et al.*, 1993; Cook *et al.*, 1989). Based on the known actions of glucocorticoids and RA with respect to regulation of gene expression, it is likely that the induction of GNMT is the result of increased transcription. In support of this hypothesis, we have found that pretreatment of hepatoma cells with actinomycin D prevented GNMT induction by DEX and RA, and abundance of the protein appears to reflect changes in its synthesis rate⁸. However, GNMT has not been reported to contain a retinoic acid- nor a glucocorticoid-response element in its promoter region (Ogawa *et al.*, 1998). Therefore, it is likely that additional intracellular signals are required to directly mediate regulation of GNMT expression. Nonetheless, we cannot exclude the possibility that increases in

phosphorylation of the protein has a role in increasing GNMT activity, particularly as glucagon and glucocorticoids can exert their action via alterations in protein kinase activity.

Homocysteine concentrations reflect the collective balance between production, from SAM-dependent transmethylation reactions, remethylation to methionine, and catabolism via the transsulfuration pathway. As has been reported (Jacob *et al.*, 1998; Jacob *et al.*, 2001), we also found that a diabetic state was characterized by a reduction in circulating homocysteine levels. This change in homocysteine homeostasis appears to reflect an increase in the activity of BHMT and the abundance of CBS, even though the activity of MS was reduced. Similarly, previous diabetes/hyperglucagonemia studies have attributed the hypohomocysteinemia to up-regulation of CBS and γ -cystathionase, whereas the activity of hepatic BHMT and MS were not significantly altered (Jacob *et al.*, 1998; Jacob *et al.*, 2001; Ratnam *et al.*, 2002). However, in the absence of flux studies it is difficult to determine homocysteine catabolism, because CBS is regulated allosterically by other factors such as SAM, a known allosteric stimulator of CBS activity (Finkelstein and Martin 1984). In support of the increase in CBS abundance that we reported, we also found that the hepatic concentration of SAM was elevated, in agreement with previous studies using hyperglucagonemic rats (Jacobs *et al.*, 2001). Elevations in hepatic SAM levels may be the result of increased expression of methionine adenosyltransferase, as it is up regulated by glucocorticoids (Gil *et al.*, 1997). An increase in hepatic SAM concentrations would also be expected to reduce 5-CH₃-THF levels as a result of allosteric inhibition of MTHFR (Jencks and Matthews 1987; Kutzback and Stokstad 1967). The lack of 5-CH₃-THF acting as an inhibitory ligand for GNMT (Wagner *et al.*, 1985), like phosphorylation, may be a contributing factor for the increase in its activity in diabetic rats. An interesting aspect of our

work was that the administration of RA to diabetic rats prevented CBS induction and SAM accumulation, and partially reduced the activity of BHMT, whereas MS activity was unaffected. In contrast to diabetes, RA alone had no effect on CBS abundance or the activity of BHMT and MS, although we have found in previous studies that RA does increase MS when provided for a longer period of time (Ozias and Schalinske 2003). However, it should be noted that the activity and abundance of GNMT remained elevated in rats treated with RA. This further illustrates the need for metabolic flux measurements under in vivo conditions to determine the outcome of these interactions.

The implications of these results for humans are profound, as diabetes and its complications are a significant health problem and the use of pharmacological retinoid compounds has risen dramatically in recent years (Wysowski *et al.*, 2002). In addition to folate, optimal metabolism of methyl groups and homocysteine is dependent on a number of B-vitamins including B₁₂, B₆, and riboflavin, as well as an adequate source of methyl groups such as methionine and choline. Moreover, it is well known that the human population has a significant prevalence of polymorphic enzymes important in methyl group, homocysteine, and folate-dependent one-carbon metabolism (Loktionov 2003). Taken together, these nutritional, genetic, and hormonal factors underscore the need for understanding the relationship between these metabolic pathways and diabetes. As most type I diabetics control their disease by the use of insulin, there nonetheless are likely many individuals that do not adequately monitor their condition. Moreover, many of the changes noted in our study were recently demonstrated in a type II diabetes (Zucker diabetic fatty rat) model, including an increase in hepatic CBS, BHMT, and SAM (Wijekoon *et al.*, 2004).

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Footnotes

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² Nieman, K. M., Rowling, M. J., Garrow, T. A., and Schalinske, K. L. (2004) Modulation of methyl group metabolism by streptozotocin-induced diabetes and all-*trans*-retinoic acid. *J. Biol. Chem.* 279: 45708-45712.

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⁴ Research associate, Department of Biological Sciences, University of Notre Dame.

⁵ Associate Professor, Department of Food Science and Human Nutrition and the Division of Nutritional Sciences, University of Illinois.

⁶ The abbreviations used are: SAM, S-adenosylmethionine; GNMT, glycine *N*-methyltransferase; SAH; S-adenosylhomocysteine; 5-CH₃-THF; 5-methyltetrahydrofolate; MS, methionine synthase; BHMT, betaine-homocysteine *S*-methyltransferase; CBS, cystathionine β-synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; RA, all-*trans*-retinoic acid; DEX, dexamethasone; STZ, streptozotocin; PMSF, phenylmethylsulfonyl fluoride; Me₂SO, dimethyl sulfoxide; HPLC, high pressure liquid chromatography.

⁷ Knoblock, V.E., Nonnecke, E.B., Rowling, M.J. and Schalinske, K.L., unpublished data.

⁸ Rowling, M.J., Nieman, K.M. and Schalinske, K.L., unpublished data.

Figure Legends

Figure 3.1. Hepatic methyl group, homocysteine, and folate-dependent one-carbon

metabolism. Methyl groups, as S-adenosylmethionine (SAM), are utilized in numerous transmethylation reactions ($X \rightarrow X\text{-CH}_3$), such as the methylation of nucleic acids, phospholipids, and neurotransmitters. Following donation of its methyl group, SAM is converted to S-adenosylhomocysteine (SAH) which is subsequently hydrolyzed to homocysteine. The irreversible catabolism of homocysteine via transsulfuration occurs through the activity of cystathionine β -synthase (CBS) and γ -cystathionase. Alternatively, homocysteine can be remethylated by the folate-independent enzyme, betaine-homocysteine-S-methyltransferase (BHMT), or the folate-dependent enzyme, methionine synthase (MS). Methyl groups from the folate-dependent one-carbon pool for homocysteine remethylation originate primarily from serine and the subsequent action of 5,10-methylenetetrahydrofolate reductase (MTHFR), the enzyme that irreversibly reduces the methylene group of 5,10- CH_2 -THF to 5- CH_3 -THF, as opposed to its use in thymidylate synthesis. Glycine N-methyltransferase (GNMT) regulates the supply and utilization of methyl groups. As shown by the dashed line, SAM is an allosteric inhibitor of MTHFR, whereas 5- CH_3 -THF is an allosteric inhibitor of GNMT activity. Additional abbreviations used are: DMG, dimethylglycine; THF, tetrahydrofolate.

Figure 3.2. Induction of hepatic glycine-N-methyltransferase (GNMT) activity and abundance in streptozotocin (STZ)-mediated diabetic and all-*trans*-retinoic acid (RA)-treated rats. Diabetic (single dose of STZ, 60 mg/kg body weight) rats were treated daily with RA (30 $\mu\text{mol/kg}$ body weight) for 5 d. Liver samples were removed and the activity

and abundance of GNMT was determined as described under Experimental Procedures. **A**, GNMT enzyme activity in diabetic and non-diabetic rats following administration of RA or corn oil. Data are expressed as means \pm S.E. ($n = 6$) and bars denoted with different letters are significantly different ($p < 0.05$). **B**, GNMT protein abundance in diabetic and non-diabetic rats following administration of RA or corn oil. A monoclonal GNMT antibody (22) was used for Western blot analysis and a representative immunoblot is shown. Data are expressed as means \pm S.E. ($n = 3$) and bars denoted with different letters are significantly different ($p < 0.05$).

Figure 3.3. Regulation of glycine *N*-methyltransferase (GNMT) expression in rat

hepatoma and pancreatic cell lines. Rat hepatoma H4IIE (*upper panel*) and pancreatic

AR42J (*lower panel*) cells were treated with the following: 0.01% Me₂SO (control, *lane 1*),

10 μ M all-*trans*-retinoic acid (RA, *lane 2*), 0.1 μ M dexamethasone (DEX, *lane 3*), or both RA

and DEX (*lane 4*). A parallel group of cells were preincubated with 100 nM insulin for 24 h

prior to their respective treatments (*lanes 5-8*). Following a 72-h incubation period, cells

were lysed and used to determine GNMT abundance via Western blotting as described under

“Experimental Procedures”.

Figure 3.4. Plasma homocysteine concentrations in streptozotocin (STZ)-mediated

diabetic and all-*trans*-retinoic acid (RA)-treated rats. Plasma samples from the same rats

as described for Fig. 1 were obtained for the determination of total homocysteine

concentrations by HPLC and fluorometric detection as described under “Experimental

Procedures". Data are expressed as means \pm SEM ($n = 6$); bars with different letters are significantly different ($p < 0.05$).

Figure 3.5. The abundance of cystathionine β -synthase (CBS) was elevated in streptozotocin (STZ)-mediated diabetic rats and abrogated following treatment with all-*trans*-retinoic acid (RA). Hepatic tissue samples from the same rats as described for Fig. 1 were used to determine CBS abundance by Western blot analysis as described under "Experimental Procedures". A representative immunoblot is shown above the bar graph. Data are means \pm S.E. ($n = 3$); bars denoted with different letters are significantly different ($p < 0.05$).

Tables & Figures

Table 3.1

Cumulative weight gain and blood glucose concentrations in rats treated with streptozotocin (STZ) and/or all-trans-retinoic acid (RA)

Values are expressed as mean \pm S.E. ($n = 6$). Values within a column with different letter superscripts are significantly different, $p < 0.05$.

	Weight gain <i>g</i>	Blood glucose <i>mg/dL</i>
Control	77 ± 2^a	147 ± 17^a
STZ	18 ± 3^b	539 ± 19^b
RA	73 ± 1^a	165 ± 17^a
STZ + RA	26 ± 5^b	435 ± 51^c

Table 3.2

Activity of remethylation enzymes methionine synthase (MS) and betaine-homocysteine S-methyltransferase (BHMT) in the livers of rats treated with streptozotocin (STZ) and/or all-trans-retinoic acid (RA)

Values are expressed as mean \pm S.E. ($n = 6$). Values within a column with different letter superscripts are significantly different, $p < 0.05$.

	BHMT <i>units/mg protein</i>	MS <i>pmol/min · mg protein</i>
Control	40 ± 3^a	86 ± 11^a
STZ	132 ± 11^b	41 ± 5^b
RA	51 ± 4^a	119 ± 19^a
STZ + RA	100 ± 8^c	36 ± 6^b

Table 3.3

Hepatic concentrations of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) from rats treated with streptozotocin (STZ) and/or all-trans-retinoic acid (RA)

Values are expressed as mean \pm S.E. ($n = 5-6$). Values within a column with different letter superscripts are significantly different, $p < 0.05$.

	SAM	SAH	SAM / SAH
	<i>nmol/g liver</i>		
Control	42.7 \pm 2.3 ^a	34.9 \pm 2.1 ^a	1.24 \pm 0.08 ^a
STZ	114.9 \pm 22.5 ^b	66.3 \pm 10.2 ^b	1.77 \pm 0.34 ^a
RA	38.9 \pm 3.1 ^a	34.5 \pm 4.1 ^a	1.20 \pm 0.15 ^a
STZ + RA	43.6 \pm 3.7 ^a	40.3 \pm 4.3 ^a	1.14 \pm 0.12 ^a

Figure 3.1

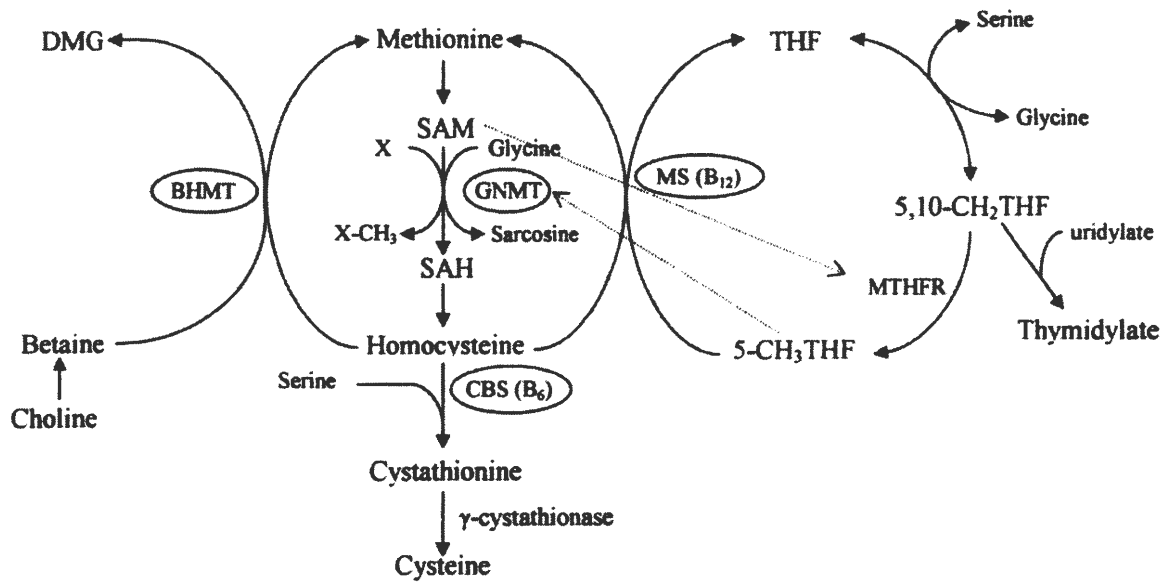
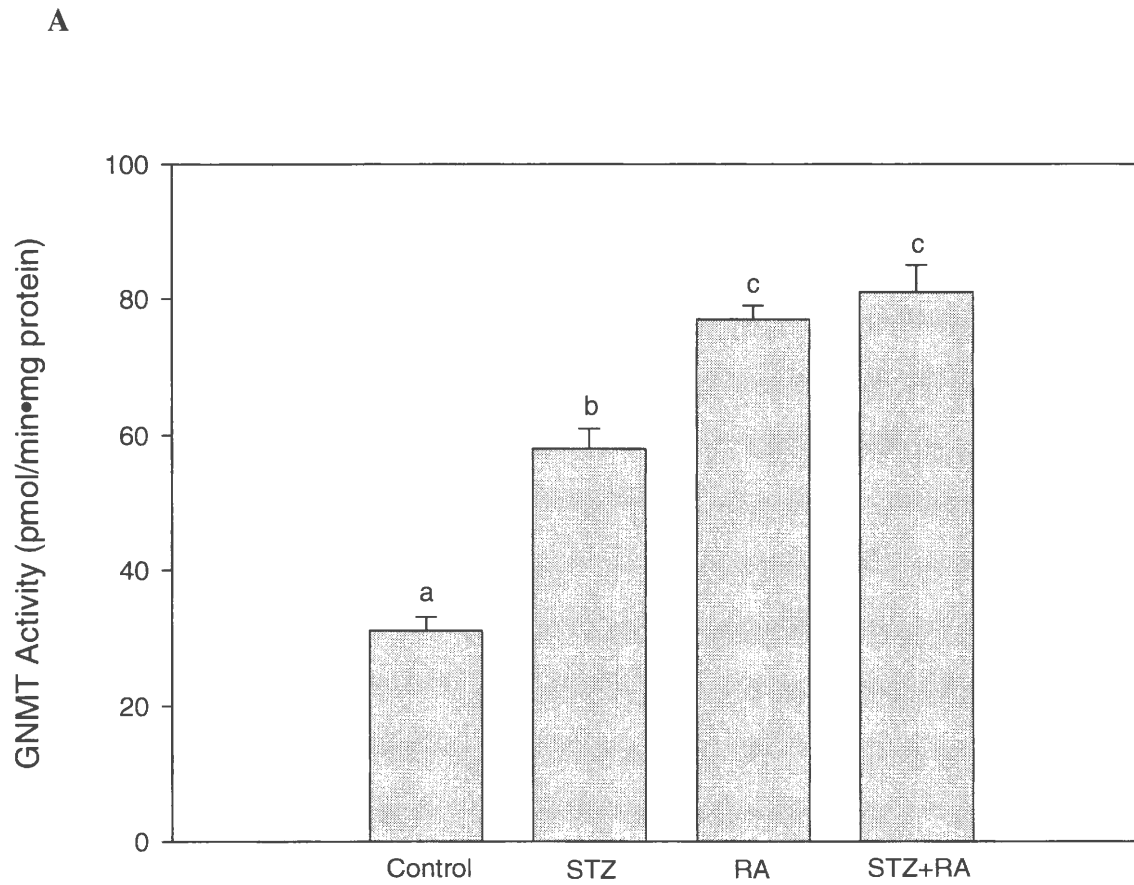


Figure 3.2



B



STZ:	-	+	-	+
RA:	-	-	+	+

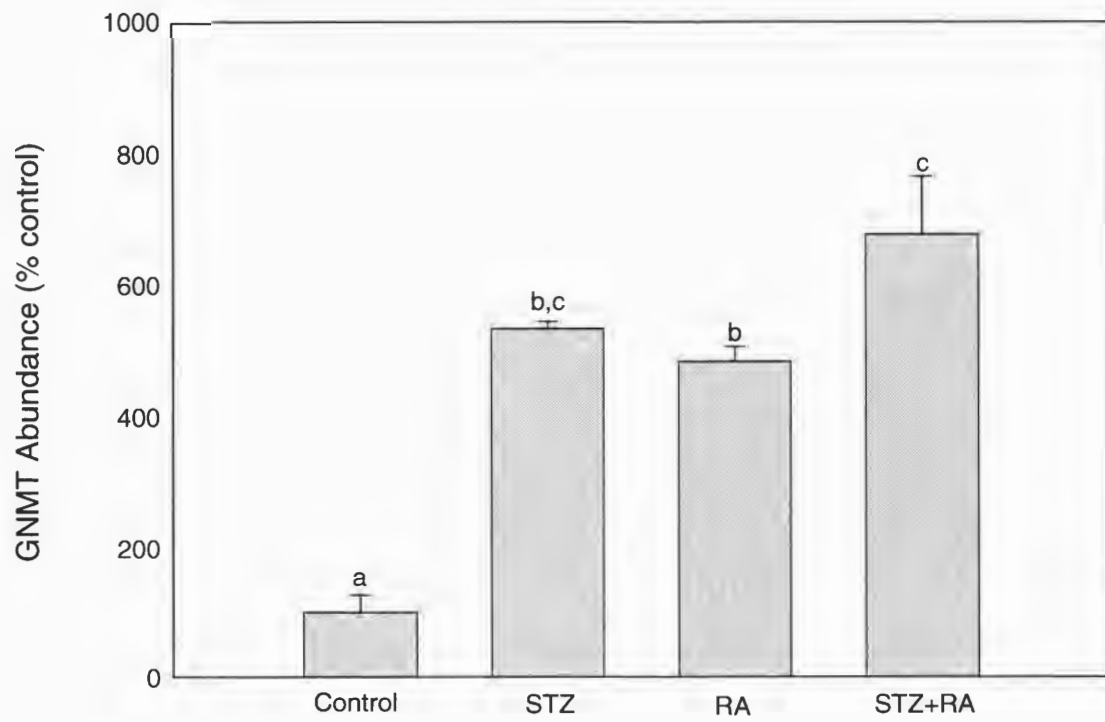


Figure 3.3

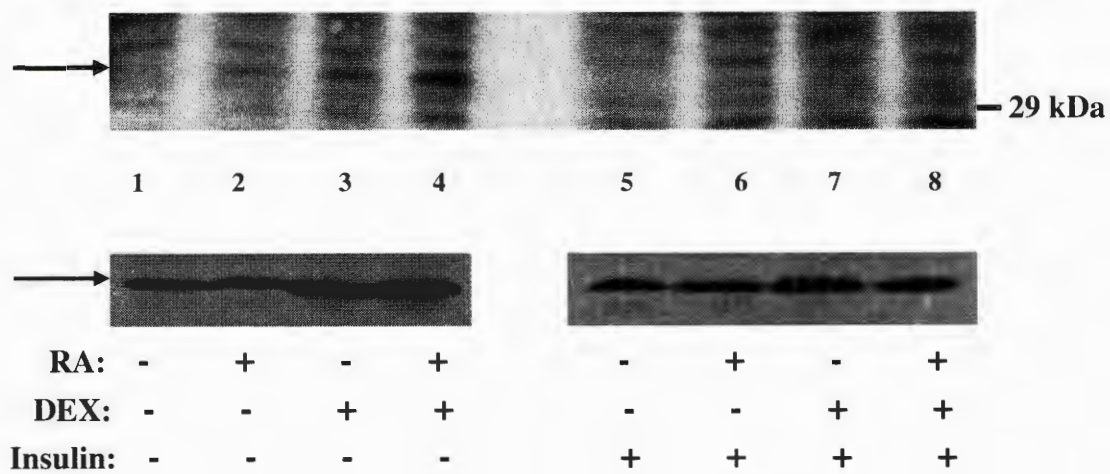


Figure 3.4

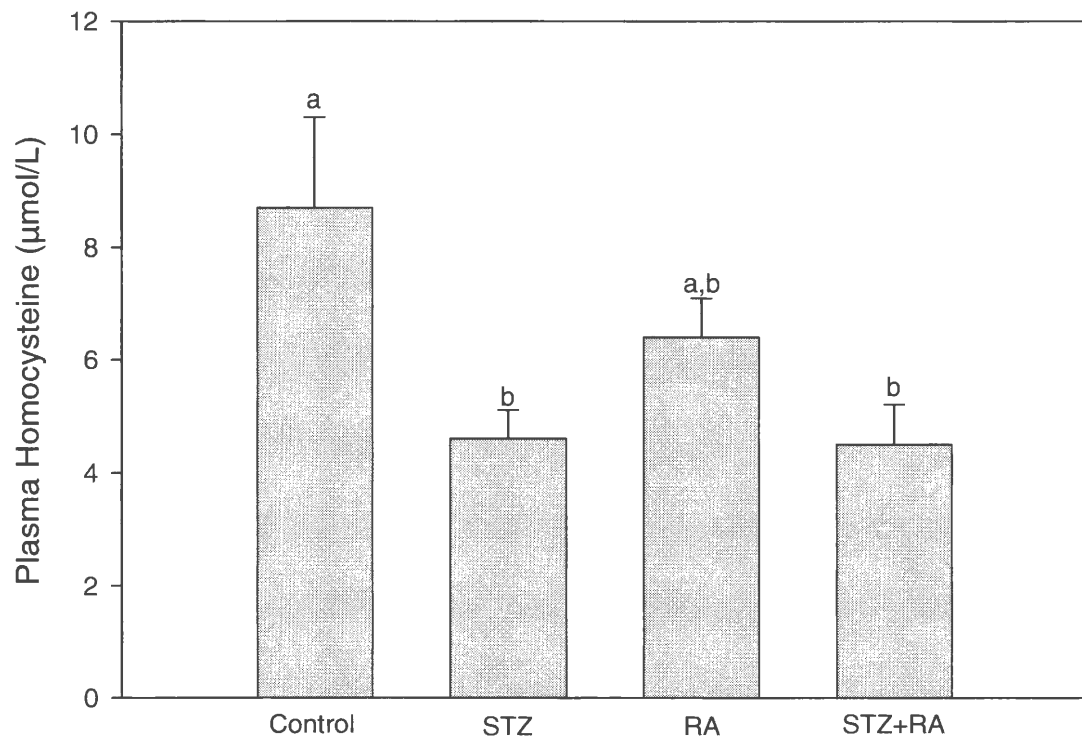
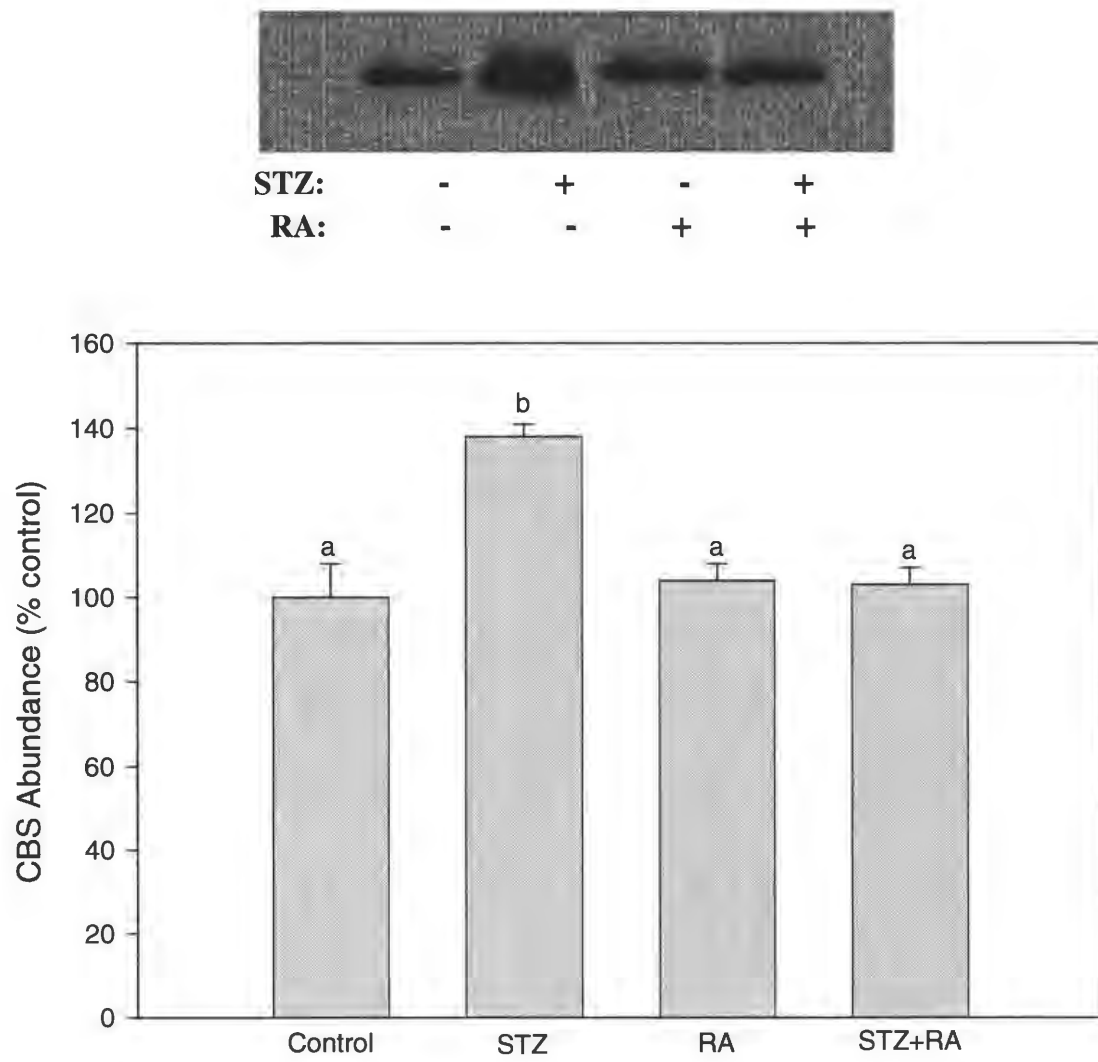


Figure 3.5



CHAPTER 4 - GENERAL CONCLUSIONS

Folate, homocysteine, and one-carbon metabolism function in concert to provide activated one-carbon units in the modification and synthesis of essential biological compounds. Therefore, interruptions in these vital pathways may have serious implications in health and disease. GNMT is a key regulatory mechanism in the supply of methyl groups for SAM-dependent transmethylation reactions, whereas inappropriate activation of this enzyme may lead to loss of methyl groups and in turn pathological conditions.

In this thesis we have further elucidated modifications in methyl group metabolism in a streptozotocin-induced diabetic state, characterized by reduced circulating insulin and elevated counterregulatory hormones, essentially a gluconeogenic state. RA has also been identified as having the ability to induce a gluconeogenic state by increasing the expression of key enzymes involved, and GNMT has been shown to be activated during gluconeogenic conditions (Pan *et al.*, 1990; Shin and McGrane 1998). The study revealed an induction of hepatic GNMT activity and abundance in a diabetic state. The induction of GNMT may be a result of excess SAM inhibiting MTHFR which decreases 5-CH₃THF, and alleviates negative allosteric regulation of GNMT. However, RA administration prevented elevated SAM accumulation in diabetic rats. The greatest elevation in the abundance was in diabetic rats treated with RA, indicating GNMT may be regulated at the level of transcription, translation, or post-translation due to reduced protein degradation. Further, we have shown in cell culture that induction of GNMT followed by treatment with glucocorticoids, which tend to be elevated in a diabetic state, was prevented by pretreatment with insulin.

We have also shown that a diabetic state results in reduced circulating homocysteine concentrations which may be due to increased transsulfuration and remethylation. CBS

which commits homocysteine to transsulfuration revealed induction of the protein in a diabetic state. A diabetic state also caused an increase in the activity of the folate-independent remethylation enzyme, BHMT. This reduction in homocysteine has been evident in other studies as a result of increased transsulfuration; however remethylation enzymes have not been altered in previous reports (Jacobs *et al.*, 2001).

Approximately 5.9% of the U.S. population is affected by diabetes (Franz 2000). The results reported in this thesis may have significant implications for complications associated with the disease specifically in those individuals that use retinoids therapeutically. The therapeutic use of retinoids has also increased approximately 2.5-fold in the last decade (Wysowski *et al.*, 2002) further indicating a need to understand completely, the ways in which diabetes effects methyl group metabolism.

Many questions remain and need to be addressed by future research. First, the mechanism by which GNMT is up-regulated is still unknown. Based on how glucocorticoids and RA act leads us to believe regulation occurs at the level of transcription. However, there is currently no known RA- or glucocorticoid-response element on the promoter region of GNMT (Ogawa, *et al.*, 1998). Further research to determine if these alterations emerge as a result of increased circulating counterregulatory hormones or lack of insulin, will continue to define the effect of a diabetic state on these metabolic pathways. Secondly, further research needs to be directed at determining if folate status alters the effects seen in a diabetic state. As mentioned previously, 5-CH₃THF provides allosteric regulation for GNMT. It would be interesting to investigate modulation of methyl group metabolism in a folate-deficient diabetic model, as well as a diabetic model supplemented with folate. Finally, it will be imperative to evaluate whether these perturbations in methyl group metabolism can be

prevented by insulin treatment. Studies in cell culture would indicate so, but further research should be directed to the possibly prevention with insulin *in vivo*.

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