2010

Genistein mediates perturbations in one-carbon metabolism during diet-induced folate deficiency

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by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Nutritional Sciences

Program of Study Committee:
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Iowa State University

Ames, Iowa

2010

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LIST OF ABBREVIATIONS

SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
MAT	Methionine adenosine transferase
GNMT	Glycine N-methyltransferase
BHMT	Betaine homocysteine methyltransferase
PEMT	Phosphatidylethanolamine N-methyltransferase
SHMT	Serine hydroxymethyltransferase
CBS	Cystathionine β-synthase
CGL	Cystathionine γ-lyase
GAMT	Guanidinoacetate methyltransferase
AGAT	L-arginine: glycine amidinotransferase
MS	Methionine synthase
DHF	Dihydrofolate
THF	Tetrahydrofolate
MTHFR	Methylene tetrahydrofolate reductase
MTA	Methylthioadenosine
Dnmt	DNA methyltransferase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
SNP(s)	Single nucleotide polymorphisms
HFE	Human hemochromatosis gene

NTD(s)	Neural tube defects
HRT	Hormone replacement therapy
DES	Diethylstilbestrol
CNS	Central nervous system
CVD	Cardiovascular disease
CpG	Cytosine-phosphate-guanine
BMI	Body mass index
ER-α	Estrogen receptor alpha
ER-β	Estrogen receptor beta
dUMP	Deoxyuridine monophosphate
dTMP	Deoxythymidine monophosphate
DFE	Dietary folate equivalent
ERK	Extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
eNOS	Endothelial nitric oxide synthase
AP-1	Activator protein 1
TLR	Toll-like receptor
VSMC	Vascular smooth muscle cells
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
p53	Tumor protein 53
p21	Cyclin-dependent kinase inhibitor 1
p16	Cyclin-dependent kinase inhibitor 2A

p38	Mitogen-activated protein kinases
SLE	Systemic lupus erythematosus
AF-2	Activation function 2 domain
CDK	Cyclin-dependent kinases
VDR	Vitamin D receptor
EGFR	Epithelial growth factor receptor
RXR	Retinoid X receptor
LNCaP	Human androgen-sensitive prostate adenocarcinoma cells
HepG2	Human hepatocellular carcinoma cells
BRCA1	Breast cancer type 1 susceptibility protein
HAT	Histone acetyltransferase
hTERT	Human telomerase reverse transcriptase promoter

ABSTRACT

Homeostasic balance of one-carbon metabolism is highly dependent upon derivatives of folate and is vital for the prevention of multiple disease states, including vascular disease, birth defects, neurological disorders, and cancer. Homocysteine, a non-protein forming amino acid, accumulates in the absence of 5-methyltetrahydrofolate and represents both a marker for reduced cellular methylation potential and elicitor of pathogenesis (Cattaneo, 1999). In addition to cofactors like folate, vitamin B_6, B_{12}, and B_2, various bioactive compounds are believed to influence the same cellular processes either directly or indirectly (Strain et al., 2004). The isoflavone genistein exhibits estrogen-like properties and has been implicated in the prevention of various diseases, including those involving hyperhomocysteinemia (Marini et al., 2009). Genistein has been shown to stably alter DNA methyltransferase and histone acetyltransferase activities independently of folate (Dolinoy et al, 2006; Li et al., 2009). This study was conducted to determine if genistein could modulate one-carbon metabolism during diet-induced folate deficiency. Specifically, we aimed to determine whether genistein is able to prevent hyperhomocysteinemia caused by moderate folate deficiency and classify any changes in relevant enzyme function, including those related to transmethylation, remethylation, and transsulfuration pathways, as well as alterations in genomic DNA methylation patterns. Administration of genistein (300 mg/kg diet) reduced plasma homocysteine concentrations in rats fed a folate-deficient diet in three independent studies without consistent alterations in enzyme activity or expression. Attenuation of plasma homocysteine was similar and occurred independently across each study at 10, 24, and 59 d of supplementation. There were no differences in genomic DNA methylation patterns

or measured hepatic transmethylation, remethylation, and transsulfuration enzymes as determined by analysis of enzyme expression and activity. Future research is warranted to determine the specific actions of genistein related to its ability to mediate one-carbon metabolism during moderate folate deficiency.

CHAPTER 1. GENERAL INTRODUCTION

Thesis Organization

The thesis is composed of three chapters that include a general introduction, the description of experiments, and general conclusions. Chapter one consists of a general introduction describing the overall research question addressed and a literature review that encompasses relevant research regarding one-carbon metabolism, the importance of homocysteine, epigenetics, and how genistein may interacts with these systems. The experiments are discussed in chapter two, including methods and results for related studies. A general conclusion of the experiments is given in chapter three. References cited in all three chapters follow Chapter 3.

Description of Research Questions

The research aim for this thesis was to examine the effects of dietary genistein on perturbations in one-carbon metabolism due to diet-induced folate deficiency. Populations regularly consuming soy foodstuffs containing isoflavones exhibit a lower incidence of cardiovascular disease and estrogen-responsive cancers (e.g., breast and prostate cancers) (Sacks et al., 2006). However, many other dietary constituents (i.e., macro/micro-nutrients and bioactive compounds) vary between cultures, as do lifestyles and genetic predispositions to disease (Fang et al., 2007). Citizens of industrialized countries, like the United States, consume on average a fraction of the soy-based food products compared to people living in countries such as China and Japan. Individuals migrating from these countries to the United States are at increased risk for developing

disease (i.e., obesity, diabetes, CVD, and certain cancers), possibly related to changes in dietary intake (Penza et al., 2007).

Population data attributing isoflavone intake to health promotion are poorly defined, as humans do not consume pure dietary constituents nor are diets constant within a given population. The purified isoflavone genistein, a 17-β-estradiol-like molecule, has shown various biological effects in cell-culture and animal models. Genistein displays paradoxical activity, as an agonist and antagonist to endogenous estrogen-receptors (e.g., ER-α and ER-β). Genistein has been shown to influence a diverse range of cellular pathways where dosage, model, and duration of treatment can produce variable, sometimes polar effects (Sacks et al., 2006).

One-carbon metabolism includes a series of interrelated pathways that are responsible for the production and regulation of proteins, hormones, phospholipids, and nucleic acids. S-adenosylmethionine (SAM) is the major biological methyl-donor for all organisms (Wagner, 1995). Upon transferring a methyl group, SAM forms homocysteine, a non-protein forming amino acid, which can accumulate if not remethylated to back methionine or degraded to cysteine via the transsulfuration pathway. Hyperhomocysteinemia is associated with various pathologies, including cardiovascular disease, neural tube defects, and certain cancers (Aguilar et al., 2004). Homocysteine represents a marker of cellular methyl-potential, which influences among other things DNA-methylation, an epigenetic mechanism that modulates transcription, translation, differentiation, proliferation, and apoptosis (Fux et al., 2005). The folate coenzyme, 5-methyltetrahydrofolate, is responsible for remethylating homocysteine to methionine in concert with the vitamin B_{12} dependent enzyme methionine synthase.

Chronic folate deficiency poses severe metabolic consequences including development of various diseases due to its role in nucleic acid synthesis, DNA methylation, and methionine metabolism (Fux et al., 2005).

Genistein has been shown to influence DNA methylation in estrogen-responsive tissues. However, the specific interactions are not completely understood, particularly in regards to folate metabolism, which governs the activity of the DNA-methyltransferase (i.e., Dnmt 1, 3a, and 3b) enzymes (Day et al., 2002).

My research aim was to investigate the specific actions of pure genistein during moderate diet-induced folate deficiency. Our diet model incorporated an amount of genistein equivalent to what an individual could consume with either a diet rich in soy foodstuffs or by supplementation. Using a folate-restricted system, without severe interventions, such as antibiotic administration (e.g., sulfamethoxazole) or choline restriction, we modeled a subset of the population exhibiting perturbations of folate metabolism due to either inadequate folate intake or altered metabolism, common in individuals with polymorphisms of genes regulating folate metabolism (Bailey and Gregory, 1999).

Literature Review

Folate Metabolism

Folate (vitamin B_9) is a common name that describes a diverse group of molecules derived from tetrahydrofolate (THF). One-carbon metabolism describes reactions utilizing folate coenzymes to donate or accept one-carbon units (Bailey and Gregory, 1999). Fully oxidized folate or folic acid (pteroylmonoglutamate), is comprised of a 2-amino-4-hydroxy-pteridine moiety bound to p-aminobenzoylglutamic acid by a methylene group (Wagner, 1995) (Figure 1). Plants synthesize a reduced form of folate (7,8-dihydrofolate), which is sensitive to food processing methods such as boiling. In the animal, the majority of tissue folates are polyglutamate species. Monoglutamate species are not retained within the cell and are transported out of the cell to maintain normal cellular folate metabolism, which is under tight control (Stipanuk, 2006). Folate monoglutamates are absorbed in the small intestine by a folate transmembrane protein, encoded by the reduced folate carrier gene (RFC-1). Although protein-mediated absorption is most common, folate monoglutamates can diffuse across the enterocyte at high concentrations during periods of supplementation with folic acid. Whole foodstuffs, such as vegetables, when digested provide in the polyglutamate form of folate. In the small intestine, the brush boarder enzyme γ-glutamylhydrolase cleaves polyglutamates into monoglutamates prior to absorption across the enterocyte. Although the enterocyte can metabolize folate, the majority of absorbed monoglutamates remain unchanged as reflected in the portal circulation. In mammals, cells are unable to transfer folate molecules containing more than two glutamate moieties. The major circulating folates are pteroylmonoglutamates, of which the primary form is 5-methyltetrahydrofolate, the

major cytosolic form in mammalian tissues (Wagner, 1995). Certain tissues exhibit

affinity for folate uptake, including the proximal tubule of the kidney, the choroid plexus,

the placenta, and erythropoietic cells. This phenomenon, mediated by folate-binding

proteins, highlights both functional and protective roles folate provides in specific tissues.

The folate coenzyme pool reflects the quality of ingested folate and also the rate of folate

loss through urine and feces (Bailey and Gregory, 1999).
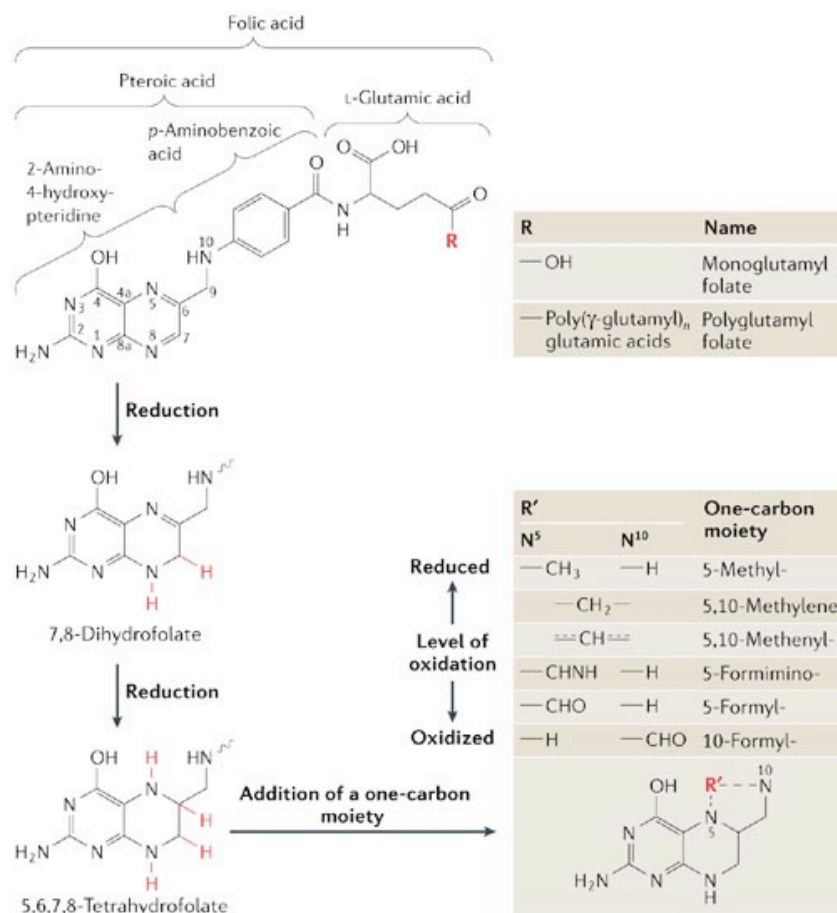
Figure 1.1. Cellular folate metabolites. Taken from Blom et al., 2006.

In industrialized countries, humans consume an average of 5-10 mmoles of methyl groups daily from dietary folates (Niculescu and Zeisel, 2002). The most common sources of dietary folate include vegetables (i.e., spinach, asparagus, peas, broccoli, beets), fortified grain products (i.e., cereals, bread, pasta), fruits (i.e., oranges, cantaloupe, grapefruit, strawberries), and organ meats (e.g., liver). The recommended daily folate requirement of 400 μg, based on the Dietary Reference Intakes (DRIs) established by the Institute of Medicine (National Academy of Sciences), satisfies the folate requirement for 97-98% of all healthy individuals. Dietary folates are ingested and absorbed in various forms. Therefore, the RDA for folate is based on an equilibration standard called the Dietary Folate Equivalent (DFE), where 1 DFE is equivalent to 1 μg of food folate or 0.6 μg of folic acid derived from supplements or fortified foods. The dietary intake range for folate (μg DFE/day) considers age and physiological status (Table 1).

Table 1.1. Range of dietary folate equivalents (μg DFE/day) based on age and physiological status (Bailey and Gregory, 1999; Food and Nutrition Board, 2000).

Age Months (mo) and Years (y)	Males and Females (μg DFE/day)	Pregnancy (μg DFE/day)	Lactation (μg DFE/day)
0-6 mo	65		
7-12 mo	80		
1-3 y	150-300	*	*
4-8 y	200-400	*	*
9-13 y	300-600	*	*
14-18 y	400-800	600-800	500-800
≥ 19 y	400-1000	600-1000	500-1000

Folate Fortification

In 1998, the FDA required that folic acid be added through fortification to cereals, flours, rice, pasta, and other grain products in the United States; there was overwhelming evidence that supplementation with 800 μg of folic acid largely negated neural tube defects (NTDs) including spina bifida and anencephaly in the offspring of women of child-bearing age (Beaudin and Stover, 2007). Further impetus to the initiative was realized because of the general knowledge that women often do not realize they are pregnant until weeks after conception and neural tube closure occurs at 4 weeks into pregnancy. The worldwide incidence of NTD(s) ranges from 0.5 to 60 per 10,000 live births (Beaudin and Stover, 2007). The association between NTD(s) and folate status was discovered in the 1960s when Hibbard and colleagues found higher concentration of formiminoglutamic acid in the urine of mothers birthing children with CNS defects, a biomarker of perturbed folate metabolism (Johnstone et al., 1965; Beaudin and Stover, 2007). Data from the National Health and Nutrition Examination Survey have documented a substantial increase in population blood folate levels between 1988-1994 and again from 1999-2000 (McDowell et al., 2008). As a parameter, serum folate is a reflection of recent folate intake, whereas erythrocyte folate represents long-term folate status as tissue folate pools turn over roughly every 100 days, as do erythrocytes. Unfortified populations exhibit plasma folate concentrations in the range of 10-30 ng/ml (Stipanuk, 2006). In 2006, the median erythrocyte folate concentration in individuals over 4 years of age was 266 ng/ml and serum folate concentration was 12.2 ng/ml. In 2006, the prevalence of folate deficiency, defined as either an erythrocyte folate concentration < 140 ng/ml or serum concentrations < 3 ng/ml was found to be 4.5% and 0.5% respectively in women of child-bearing age (McDowell et al., 2008). Folate

fortification has resulted in a 19-38% reduction in NTD(s) cases in the United States. However, within certain subsets of the population, including Hispanic populations, dietary fortification has not substantially reduced the risk of NTD(s). Other groups at risk of developing folate deficiency include the morbidly obese (BMI > 50), the elderly, alcoholics, celiacs, and those with polymorphic variations in genes regulating folate metabolism (Matsuo et al., 2005).

Folate Fortification Risks

Folate supplementation also poses risks in populations residing in certain geographical areas, such as the malaria endemic regions of sub-Saharan Africa. A cohort intervention trial termed the Pemba study, conducted in Pemba (Tanzania) and also in Nepal, aimed to reduce cases of anemia in children by providing folic acid and iron supplementation (Sazawal et al., 2006). Although the prevalence of anemia was reduced in the child population of Nepal, the trial in Pemba was forfeited due to increased morbidity and mortality within the study group. Further investigation concluded that malaria, endemic in Tanzania and non-existent in Nepal, was benefiting from bolus intakes of folic acid and iron, enhancing DNA turnover and, subsequently increasing parasitemia (Sazawal et al., 2006).

In the industrialized nations, where both food and supplemented folates are easily accessed, unknown side effects may exist, particularly related to cancer development and progression. Many chemotherapeutic agents target folate metabolism. 5-fluorouracil, a cellular S-phase specific drug, interferes with thymidylate synthase, which requires 5,10-methylenetetrahydrofolate as a substrate, preventing the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) (Backus et al.,

2000). In doing so, neoplastic cells become apoptotic due to an inherently high rate of replication and requirement for DNA synthesis (Maruti et al., 2009). Methotrexate also interferes with folate metabolism by inhibiting dihydrofolate reductase, which converts dihydrofolate (DHF) to tetrahydrofolate (THF), blocking DNA, RNA, and ultimately protein synthesis. Folic acid supplementation may interfere with the actions of these chemotherapeutic agents and also promote cancer development by enhancing DNA turnover and proliferation (Maruti et al., 2009). However, suboptimal folate status prior to administration of these cytotoxic agents increases the risk of developing hematological and non-hematological toxicity upon treatment (Backus et al., 2000). Therefore, folate may have paradoxical effects regarding cancer development, both as a preventative and a promoter.

One-carbon Metabolism

Folate metabolism occurs in both the mitochondria and cytosol, where tetrahydrofolates are stored as polyglutamates by the enzyme folylpolyglutamate synthetase (Shane, 1989). The polyglutamyl form of THF is the central folate molecule in cellular metabolism (Bailey and Gregory, 1999). The pyridoxal phosphate (PLP)-dependent enzyme, serine hydroxymethyltransferase (SHMT), catalyzes a one-carbon transfer from serine to THF, forming 5,10-methyleneTHF and glycine. The enzyme methylenetetrahydrofolate reductase (MTHFR) irreversibly converts a potion of the 5,10-methyleneTHF to 5-methylTHF, which is utilized solely for the remethylation of homocysteine.

Cellular polyglutamate synthesis favors tetrahydrofolate as the substrate. Therefore, 5-methyl-derivatives are converted to tetrahydrofolate prior to glutamate

addition by methionine synthase or exported to the circulation to maintain cellular homeostasis. Cytosolic folate metabolism includes purine biosynthesis, methionine metabolism, and thymidylate synthesis. Within the mitochondria, numerous one-carbon metabolites and cofactors undergo formation and regulation, the major products formed being formate and glycine from serine (Beaudin and Stover, 2007). Mitochondrial folate is largely 10-formyl-THF, which is required for purine biosynthesis. Cytosolic folates are bound to either glycine-N-methyltransferase (GNMT) or 10-formyltetrahydrofolate dehydrogenase. The enzyme GNMT regulates both the supply and utilization of cellular methyl-groups by modulating the folate-dependent one-carbon supply (Rowling et al., 2002). GNMT catalyzes the methylation of glycine to sarcosine, which functions in diverting excess methyl groups away from transmethylation when cellular folate and methyl groups exceed adequacy (Rowling et al., 2002). During periods of folate deficiency GNMT activity is increased, allowing optimization of the folate-transmethylation pool (Nieman et al., 2004). GNMT activity is regulated in response to cellular 5-methylTHF concentrations, a reflection of MTHFR allosteric regulation by S-adenosylmethionine. GNMT activity is also regulated through posttranslational modification by phosphorylation (Nieman et al., 2004).

S-adenosylmethionine (SAM) is the primary methyl donor for all living organisms. In plants, SAM is required for ethylene production to mature fruit and in mammals SAM methylates guanidinoacetate to produce creatine, which functions to store high-energy phosphate. In humans, SAM serves as the methyl donor for hundreds of reactions, including the methylation of DNA, RNA, proteins, phospholipids,

neurotransmitters, and hormones and also functions to regulate cell proliferation, differentiation, and apoptosis (Lu and Mato, 2008).

Transmethylation

In a two-part reaction, methionine is converted to SAM by methionine adenosyltransferase (MAT). Initially, adenosine triphosphate (ATP) and methionine are converted to SAM and tripolyphosphate by MAT. The tripolyphosphate is hydrolyzed to orthophosphate and pyrophosphate, at which point the remaining phosphate is removed from the adenosine by a subsequent MAT reaction. The genes MAT1A and MAT2A encode the MAT enzymes II, III, and I, which are highly expressed in mammalian hepatocytes and other tissues (Prudova, 2005). Within the cell, SAM regulates both MAT1a and MAT2A expression through an unknown feedback mechanism in an opposing fashion (Grillo and Colombatto, 2008; Martinez-Chantar et al., 2003). When sufficient SAM is formed, MAT2A expression is downregulated and SAM production is decreased. The cellular availability of methionine also regulates MAT2A expression, and its restriction favors conservation of SAM via increased half-life of MAT2A mRNA through the methionine salvage pathway where methylthioadenosine (MTA), a product of transmethylation in polyamine synthesis, is recycled to methionine to maintain cellular SAM homeostasis (Martinez-Chantar, 2003).

The transmethylation pathway, which transfers one-carbon moieties from SAM to various acceptor molecules, is vital for a number of cellular processes (Figure 1.2). SAM irreversibly donates a methyl-group to a methyl-acceptor (R-CH$_3$) molecule forming S-adenosylhomocysteine (SAH), which is reversibly hydrolyzed to homocysteine by SAH-hydrolase (SAHH). The one-carbon moiety attached to the sulfur atom of methionine

serves as the donated methyl group. The high-energy sulfonium ion of SAM promotes

nucleophilic attack by the attached carbons, promoting the major SAM-dependent

reactions: transmethylation, transsulfuration, and aminopropylation (Lieber and Packer,

2002). SAM-dependent reactions are strongly inhibited by increases in cellular SAH.

Therefore, removal of SAH and homocysteine is essential to maintain normal cellular

methyl-potential (Lieber and Packer, 2002). The R-CH$_3$(s) include an array of cellular

constituents including, guanidinoacetate, phosphatidylethanolamine (PE), 5'-cytosines of

DNA, and polyamines. The formation of phosphatidylcholine (PC) from PE and creatine

from guanidinoacetate collectively consume upwards of 80% of SAM-derived methyl

groups, with the remaining 20% being divided among over 100+ reactions.
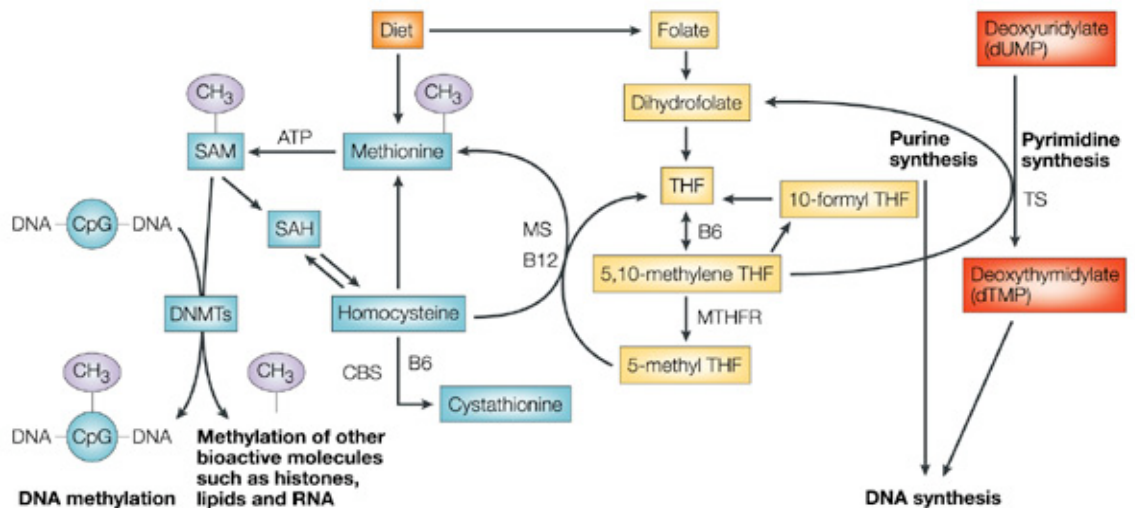
Figure 1.2. Folate and one-carbon metabolism pathway (Taken from Lamprecht and Lipkin, 2003).

Homocysteine and Disease

Vigneaud and Dyer (1932) first classified "homocysteine" and "homocystine," reduced (sulfhydryl) and oxidized (disulfide) non-protein amino acid molecules, homologous to cysteine and cystine with the addition of one methylene group (Yap, 2005). Homocysteine ($-SCH_2CH_2CH(NH_2)COOH$) can refer to both free and bound homocysteine, including homocystine, S-adenosylhomocysteine, homocysteine-cysteine, and other Homocysteine-bound proteins that form a free thiol-homocysteine upon cleavage of the connecting disulfide bond (Mudd, 2000). Total homocysteine (tHcy) includes both free and bound homocysteine and is a quantification of all homocysteine after reductive cleavage from bound constituents within a sample (i.e., urinary tHcy, hepatocyte tHcy, and plasma tHcy) (Martinez-Chantar et al., 2003). Cysteine, a conditionally essential amino acid, can be formed from methionine-derived homocysteine via the transsulfuration pathway (Yap, 2005).

Hyperhomocysteinemia is an aberrant increase in circulating plasma and serum homocysteine concentration (Cattaneo, 1999). A moderately elevated plasma homocysteine concentration (15 μmol/L) is considered to be an independent risk factor for cardiovascular disease (CVD) including, stroke, myocardial infarction, venous thrombosis, and peripheral artery disease (Khandanpour et al., 2009). Hyperhomocysteinemia is believed to exist in 15% of the general population and in over 50% of vascular patients (Spark et al., 2003). A 5 μmol/L increase in plasma homocysteine in women and men increases the risk of developing coronary heart disease by 60% and 80%, respectively (Spark et al., 2003); however, it has not been established

in humans if plasma homocysteine reflects intracellular homocysteine or the underlying pathogenesis (Cattaneo, 1999). Homocysteine has been linked to increased vascular endothelial cell dysfunction by decreasing endothelin-1 biosynthesis, increasing inflammation and promoting thrombosis by up-regulating the coagulation cascade (i.e., platelet aggregation, fibrinolysis, increased vascular endothelial growth factor expression) (Aguilar et al., 2004). Homocysteine initiates signal-transduction induced oxidative damage by upregulating metalloproteinases through the activation of mitogen-activated protein kinases (i.e., ERK, JNK, and p38) (Moshal et al., 2006). Homocysteine also induces endothelial cell dysfunction by disrupting endothelial nitric oxide synthase (eNOS) synthesis and AP-1 signaling pathways (e.g., apoptosis). The production of SAH inhibits DNA methylation in endothelial cells but not vascular smooth muscle cells (VSMC) (Huang et al., 2008); however, homocysteine activates NF-kB, stimulating the production of proinflammatory cytokines and differentiation of VSMC. Homocysteine promotes lipid/LDL oxidation through the production of hydrogen peroxide in the presence of copper and can form highly reactive –NH-CO- adducts, promoting cellular damage.

Beyond CVD, hyperhomocysteinemia is also associated with neural tube defects, low birth weight, renal disease, diabetes, neurological disorders, osteoporosis, and various forms of cancer. Epidemiological evidence relating homocysteine to chronic disease has not defined whether its elevation induces, exacerbates, or serves as a marker for disease. Acute plasma homocysteine loading (65.1 μM) in healthy men was not found to change the methyl-potential or DNA methylation status in isolated lymphocytes (Fux et al., 2005); therefore, the detrimental effects of homocysteine are more strongly

associated with chronic elevations. Men generally display higher plasma homocysteine concentrations compared to women, and although this discrepancy is reduced upon the onset of menopause, it persists in the elderly.

Although the specific mechanisms underlying homocysteine mediated CVD are not well established, multiple relationships are believed to exist related to its action as an oxidant including endothelium dysfunction, procoagulant actions (thrombosis), arterial stiffness, thickness, and immune modulation via inflammation (Strain et al., 2004). A decreased methyl potential within a cell, reflected by increased plasma homocysteine concentrations, can directly influence pathogenesis via alterations of the epigenome (discussed later). Elevated plasma homocysteine and S-adenosylhomocysteine (SAH) concentrations in elderly CVD patients coincide with leukocyte global hypomethylation (Castro et al., 2003; Strain et al., 2004).

Preventing Hyperhomocysteinemia

Although chronic elevation of homocysteine often coincides with complex pathogenesis in humans, a number of polymorphisms in homocysteine re-methylation and transsulfuration pathways exist that can interfere with optimal methyl-group metabolism (Verhoef, 1996). Deficiencies in cofactors required for transmethylation, remethylation and transsulfuration including folate (B_9), cobolamin (B_{12}), pyrodoxine (B_6), riboflavin (B_2), and the methyl-donors choline and methionine contribute to elevated homocysteine but also perturb numerous cellular reactions, which in turn, exacerbate the risk of developing chronic disease (Verhoef, 1996). Supplementation with folic acid provides the most effective approach for reducing plasma homocysteine concentrations. A folate intake of 400 μg/d provides 90% of the maximal decrease of homocysteine (Van

Oort et al., 2003). When combined with vitamin B_{12} (0.5 mg/d) an additional 7%

reduction of homocysteine over the 25% reduction by folic acid supplementation occurs,

arguing that although folic acid alone is the most effect combatant against

hyperhomocysteinemia, combination therapy may be valuable for certain populations

(Strain et al., 2004). Optimizing folate status through supplementation with folic acid

alone can mask vitamin B_{12} (cobalamin) deficiency.

Cobolamin (Vitamin B_{12})

Cobolamin includes a group of molecules with the biological activity of vitamin

B_{12}. Vitamin B_{12} is required for two major metabolic reactions. However, its absence

significantly affects the overall physiology of an organism. The coenzyme forms of

cobalamin include free Co^{1+} or cob(I)alamin, 5'-deoxyadenosylcob(III)alamin, and

methyl-cob(III)alamin. Vitamin B_{12} functions as a coenzyme for the conversion of

malonyl-CoA to succinyl-CoA by the mitochondrial enzyme methyl-malonyl-CoA

mutase during oxidative phosphorylation. In addition, vitamin B_{12} serves as a cofactor

(methyl-cob(III)alamin) for the cytosolic enzyme methionine synthase, which forms

endogenous methionine from homocysteine via the folate-dependent one-carbon pool. In

this reaction, the methyl group is transferred from the substrate, 5-methylTHF to the

vitamin B_{12}-coenzyme and finally to homocysteine to produce methionine. By donating

its methyl-group, 5-methylTHF is recycled to THF, which can be converted to 5,10-

methyleneTHF and 10-formylTHF required for thymidylate and purine synthesis. The

enzyme methionine synthase reductase (MTRR) catalyzes the methylation of

cob(II)alamin back to methyl-cob(III)alamin, restoring the activity of methionine

synthase (Beaudin and Stover, 2007). Therefore, the folate pool is cyclic and reflects

cellular requirements. Fifty to eighty percent of homocysteine is remethylated to methionine (Bailey and Gregory, 1999). Liver and muscle are the major storage sites for vitamin B_{12}. Enterohepatic reabsorption of cobalamin from the bile protects against periods of acute deficiency, as turnover rates are estimated to be low at 0.1% per day.

Cobolamin Deficiency

Vitamin B_{12} deficiency predisposes an individual to megaloblastic anemia, neurological disorders such as Alzheimer's disease, and bi-polar disorder. Subacute combined degeneration of the spinal chord, which involves deterioration of the myelin sheath, is common in upwards of 80% of individuals with clinical B_{12} deficiency. In addition to signs and symptoms, serum concentration of cobalamin and two metabolic indices of its metabolism, methyl-malonic acid (> 0.4 μM) and homocysteine (> 13 μM), are used to assess vitamin B_{12} deficiency (Andres et al., 2004).

Vitamin B_{12} deficiency (serum B_{12} < 150 pM) is believed to exist in roughly 20% of the population in industrialized countries (Andres et al., 2004). Vitamin B_{12} deficiency, common in the elderly, results from malabsorption and pernicious anemia. Gastric atrophy occurs in over 40% of individuals 80 years of age and may be caused by exposure to *Helicobacter pylori* infection, chronic antacid use, surgery, and antibiotic treatments (Andres et al, 2004). The autoimmune disease, pernicious anemia (Biermer's disease), is a major cause of cobalamin deficiency as it destroys the gastric mucosa, decreasing the production of intrinsic factor by the parietal cells of the stomach. Cobalamin is bound to haptocorrin (R-factor) in the stomach, forming a complex that travels to the small intestine where the change in pH and hydrolysis by pancreatic enzymes promote the release of the complex and binding of intrinsic factor, allowing

absorption across specialized cells of the ileum (Andres et al., 2004). Without adequate cobalamin absorption, methionine synthase cannot adequately remethylate homocysteine. If vitamin B_{12} deficiency exists in the presence of adequate folate, a deleterious phenomenon occurs know as the "methyl-trap," where the folate pool is wastefully directed towards the production of 5-methylTHF to remethylate the accumulating homocysteine, siphoning the folate pool away from other essential metabolic reactions (Beaudin and Stover, 2007).

Choline and Betaine

The enzyme betaine-homocysteine methyltransferase (BHMT) offers an alternative, folate-independent remethylation pathway (Lieber and Packer, 2002). This reaction occurs largely in the liver and kidney and is believed to remethylate up to 25% of cellular homocysteine in these tissues. SAM-dependent transmethylation inhibits the activity of BHMT, favoring the shunting of homocysteine into the transsulfuration pathway. Betaine (trimethylglycine) is derived from the oxidation of choline and forms dimethylglycine upon donation of its methyl group to homocysteine. Choline, a major dietary source of methyl groups, is a component of phospholipids (signaling molecule), is utilized in the kidney to maintain water balance, and functions as a neurotransmitter (acetylcholine) (Zeisel, 1997). Choline oxidase and betaine aldehyde dehydrogenase sequentially oxidize choline to betaine in the mitochondria. Rodents rely on BHMT for the remethylation of homocysteine much more than primates, who rely less on choline oxidase (Lieber and Packer, 2002). Betaine attributes for 0.6-1.6% of total protein in the liver of most species and in the Rhesus monkey betaine represents 10% of protein in the lens, suggesting functions beyond the remethylation of homocysteine. The protein is

highly conserved, as 88% of the gene and 94% of the amino acid sequence is paralleled between pigs and humans (Pajares and Perez-Sala, 2006). Betaine displays a protective role against hyperhomocysteinemia, as an intake of 2-6 g/day can reduce fasting homocysteine concentrations by 20% and 50% post methionine load, which folic acid alone cannot (Olthof and Verhoef, 2005). Although a diet rich in betaine and choline may adversely affect blood lipid profiles, inadequate intake of betaine and choline increases the risk of developing one-carbon metabolism-related disease (Olthof and Verhoef, 2005).

Polymorphism of Methylenetetrahydrofolate Reductase (MTHFR)

There are over 10 million single nucleotide polymorphisms (SNPs) in the human genome, and many are believed to influence the activity and function of proteins (Stover and Caudill, 2008). A polymorphism arises from an evolutionary response to environment (e.g., acute change in diet) and represents a specific form of genetic variation within the population. Examples of positively selected gene SNP(s) include the HFE gene, which likely protected populations from iron deficiency in iron-poor regions and the SNP upstream of lactase, which allows individuals to continue to consume milk throughout life (e.g., nomadic cattle herders) (Stover and Caudill, 2008). One-carbon THF coenzymes are required for multiple unique biochemical pathways within the cell. Methylenetetrahydofolate reductase (MTHFR) irreversibly catalyses the conversion of 5,10-methylenetetrahydrofolate, the primary methyl donor for nucleotide biosynthesis, to 5-methyltetrahydrofolate the primary cofactor for remethylation of homocysteine to methionine. Inborn errors in the MTHFR gene impair cellular methyl-potential due to reduced formation of 5-methylTHF. Individuals born with polymorphisms of MTHFR

are at increased risk of developing various pathologies, including colorectal cancer, stroke, heart disease, type2 diabetes, and poor reproductive outcomes (Pollex et al., 2005). The mutation at base pair position C677 replaces cytosine with thymine, ultimately forming valine in place of alanine. The change in amino acid sequence causes thermolability and reduced stability of the enzyme decreasing the activity. Both C667 homozygous (T/T) and heterozygous (C/T) polymorphisms in MTHFR occur and reduce enzyme function 70% and 35%, respectively (Hazra et al., 2009).

Polymorphic frequency of the MTHFR gene varies substantially within and across racial and ethnic groups. The homozygous C677TT variant is estimated to exist in 12% of both Asian and Caucasian populations, whereas the C677CT heterozygous variant is displayed in upwards of 50% of this population (Bailey and Gregory, 1999). Individuals of African descent display lower incidence of both homozygous and heterozygous MTHFR gene variance. The homozygous (T/T) polymorphism increases plasma homocysteine concentrations and the predicted increase is strongly correlated to observed risk of developing a stroke (Casas et al., 2005). MTHFR polymorphisms are strongly associated with the development of head and neck carcinoma (Reljic et al., 2007). Therefore, chronic disease related to one-carbon metabolism may initiate not only from dietary insufficiency but also underlying genetic predispositions (Suzuki et al., 2008).

The C677 homozygous (T/T) and heterozygous (C/T) polymorphisms affect cellular folate homeostasis by altering the complex flux of one-carbon folate derivatives (Wilcken and Wilcken, 1998). Wild-type MTHFR (C/C) erythrocyte folates exist primarily as 5-methylTHF polyglutamates, whereas in C677TT variants erythrocyte folates include up to 22% formyl-THF polyglutamates, a reflection of reduced enzyme

function (Bailey and Gregory, 1999). Previous research indicates conflicting outcomes within MTHFR variants including lower fasting plasma homocysteine concentrations in C677TT homozygotes and both positive and negative effects on erythrocyte folate concentration. Although the C677TT gene variant is believed to be the most potent of all MTHFR polymorphisms, supplementation with folic acid reduces plasma homocysteine more effectively in this variant when compared with heterozygous (C/T) or wild-type (C/C) genotypes (Bailey and Gregory, 1999). Therefore, exacerbation of plasma homocysteine concentrations and alterations in cellular methyl-potential noted in C677TT variants is highly dependent upon folate status. Optimizing folate intake may stabilize the thermolability of the mutant (T/T) enzyme, suggesting a higher folate requirement in these individuals (Ilhan et al., 2007). MTHFR (T/T) variants consuming adequate folate or folic acid supplements are less likely to develop colorectal cancer, whereas these protective effects are negated in subjects with low blood folate levels (Wilcken and Wilken, 1998). Vitamin B_{12} deficiency is strongly associated with elevated plasma homocysteine in MTHFR C677TT gene carriers (Angelo et al., 2000).

A second common polymorphism in the MTHFR enzyme occurs at base pair position 1298, where adenine is substituted with cytosine, ultimately forming alanine in place of glutamate. Approximately 10% of individuals are believed to exhibit this polymorphism (Weisberg et al., 1998). The substitution at base pair 1298 is not believed to increase plasma homocysteine concentrations. In fact, homozygous (C/C) variants may exhibit lower fasting plasma homocysteine. Nevertheless, isolated lymphocytes from individuals with the 1298CC polymorphism have a 40% reduction of MTHFR activity (Weisberg et al, 1998). The 1298CC mutation is believed to affect MTHFR signaling

through SAM, whereas the C677C/T and T/T variants modulate the catalytic ability the enzyme itself. Collectively, T/T and C/C polymorphisms steer one-carbon moieties, directed by folate derivatives to either methionine metabolism or DNA methylation, as expressed by the ability to form 5-methylTHF (Figure 1.3).
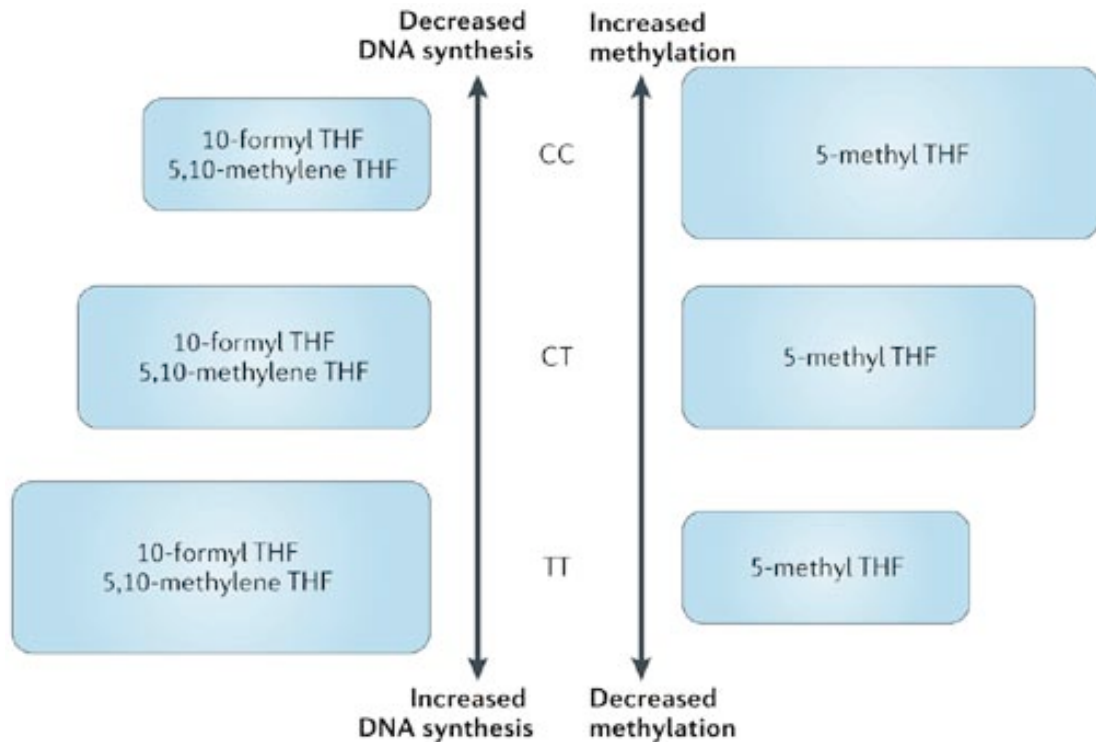
Figure 1.3. Methylenetetrahydrofolate reductase (MTHFR) polymorphisms and methylation potential (Taken from Blom et al., 2006).

Transsulfuration and Disease

The enzyme cystathionine β-synthase (CBS) metabolizes homocysteine to cystathionine, which is converted by cystathionine γ-lyase (CGL) to cysteine, ammonia, and α-ketoglutarate in a process called transsulfuration. Transsulfuration occurs primarily in the liver but not in all tissues (Finkelstein et al., 1971). Both CBS and CGL require pyridoxal 5-phosphate (B_6) as cofactors. Approximately 50% of homocysteine derived from cellular methionine is metabolized via transsulfuration. In addition to cysteine, other molecules including, taurine, glutathione, and pyruvate, may be formed via transsulfuration. The production of glutathione is dependent upon cellular cysteine availability (Cho et al., 1984). Cysteine is converted to γ-glutamylcysteine and then glutathione (γ-glutamylcysteinylglycine) through consecutive energy-dependent reactions with glutamate and glycine (Lieber and Packer, 2002).

Glutathione is also an endogenous source of cysteine and functions as a source of sulfhydryl, as an amino acid transport molecule, a cosubstrate in drug metabolism and detoxification, and as a major redox molecule within the cell (Cho et al., 1984). Cysteine deficiency, occurring protein energy malnutrition in premature infants, can deplete glutathione stores in the liver. Glutathione protects cellular membranes from lipid peroxide damage. Therefore, cysteine depletion associated with starvation or strict adherence to parenteral nutrition can disrupt cellular redox homeostasis (Cho et al., 1984).

Classic homocystinuria is a consequence of at least one mutation in the gene coding for the enzyme cystathionine β-synthase (CBS) and can perturb the transsulfuration pathway that catabolizes the carbon skeleton of homocysteine. In

addition to elevated plasma homocysteine concentrations, CBS variants display ectopic lentis, myopia, elongated limbs, and impaired cognitive development (Yap, 2005). If left untreated, advanced morbidities including scoliosis, osteoporosis, seizers, mental retardation, and thromboembolism can develop in early adulthood (Picker and Levy, 2004). Newborn and clinical screening likely underestimates the prevalence of CBS deficiency, which is of particular concern in populations exhibiting nutrient deficiencies, namely vitamin B_6, folate, and vitamin B_{12} (Yap, 2005).

Prior to the development of advanced biochemical techniques, the term homocystinuria described severely elevated urine homocysteine concentrations due to unknown errors in the enzymes regulating methionine metabolism (Mudd et al., 2000). Normal urinary excretion of homocysteine is in the range of 3.5 to 9.5 μmol/L and is considered low with an average of 6 μmol excreted per 24-hour period (Refsum et al., 1985). Therefore, homocystinuria currently relates largely to polymorphisms of CBS, which significantly elevates urinary homocysteine. In the United States, the reported incidence of homocystinuria due to CBS deficiency is 1:100,000. Over 18% of individuals diagnosed with CBS enzyme deficiency will die by 30 years of age due to cardiovascular disease (Mudd et al., 2000). The two major recessive, autosomal CBS polymorphisms are the I278T and G307S missense mutations found on exon 8 (Yap, 2005). Polymorphism I278T is the major disease causing mutation, representing 29% and 18% of mutant alleles in the United Kingdom and United States, respectively. The G307S mutation is most common in individuals of Irish decent. Over 70% of CBS mutations in the Irish population are located at G307S. The treatment regimen for CBS functional variance includes supplementation with folic acid, vitamin B_{12}, vitamin B_6, and

betaine, and in certain circumstances a protein restricted diet (e.g., methionine restricted, cysteine supplemented diet) (Picker and Levy, 2004).

Epigenetics

Epigenetics is the study of heritable changes in gene expression not caused by alterations in DNA sequence (G-C-A-T) but rather by superimposed additions to the underlying sequence. Epi," Greek for "above," describes the addition of a molecule to DNA or DNA-complex protein such as a histone or DNA that modifies transcription and ultimately the phenotype of a particular cell, tissue, and organism. Epigenetic mechanisms include but are not limited to nucleosome remodeling, DNA methylation, prion inheritance, nucleosome modification, chromosome remodeling, gene imprinting, micro RNA, and histone acetylation or ribosylation.

Environmental factors including nutritional status and exposure to stress, toxins, and infectious agents are believed to continually modify the epigenome (Stover and Caudill, 2008). Although not all epigenetic imprints are permanent, inheritance by subsequent generations may influence the ultimate susceptibility to numerous diseases depending upon the specific characteristics of the modification. DNA methylation is an epigenetic mechanism that is highly influenced by environment, particularly B-vitamin status as folate derivatives not only function in nucleotide synthesis but also in DNA methyltransferase reactions, which regulate gene expression (Ross, 2003). DNA methylation involves the addition of a methyl group to the 5' carbon of the pyrimidine nucleus of cytosine within cytosine-guanine regions of DNA. Roughly 80% of the CpG dinucleotide regions of the genome are normally methylated, which serves to suppress transcription after the formation of new DNA strands (Strain et al., 2004). It is estimated

that approximately 30% of the genome is regulated through DNA methylation (Beaudin and Stover, 2007). Methylation impedes transcription and stabilizes cellular expression (Figure 1.4). In doing so, proliferated cells maintain tissue-specific function throughout successive mitotic events. Methylation can also occur at normally unmethylated, clustered DNA regions known as CpG islands that exist at the promoter region of some genes.

DNA Methylation and Disease

Global hypomethylation or hypermethylation of regulatory regions dictates whether a gene is active or inactive and homeostatic deviations can influence disease development. DNA methylation modifies gene expression and cellular function by modifying transposable elements, the promoter regions of housekeeping genes, and the regulatory regions of imprinted genes (Dolinoy et al., 2006). The influence of environment on DNA methylation may be most crucial during key events along the life span, including gestation, lactation, and puberty due to fetal programming, high rates of cell division, and hormonal flux (Dolinoy et al., 2006). Epigenetic events are believed to occur in utero and can be modified by maternal diet (Ross, 2003). Pregnant agouti mice (YS/WffC3Hf/Nctr-A^{vy} and VY/WffC3Hf/Nctr- A^{vy}) were fed either a methyl deficient or adequate diet (i.e., methionine, betaine, choline, vitamin B_{12}, and folate). The long terminal repeat of the agouti gene is hypomethylated in the offspring of mothers fed a methyl-deficient diet and the offspring exhibit varying degrees of yellow fur, adiposity, and diseases, including cancer. Providing a diet rich in methyl groups prevents hypomethylation of the agouti gene and the offspring are born with and maintain the wild type, lean/agouti (e.g., brown coat color) phenotype.
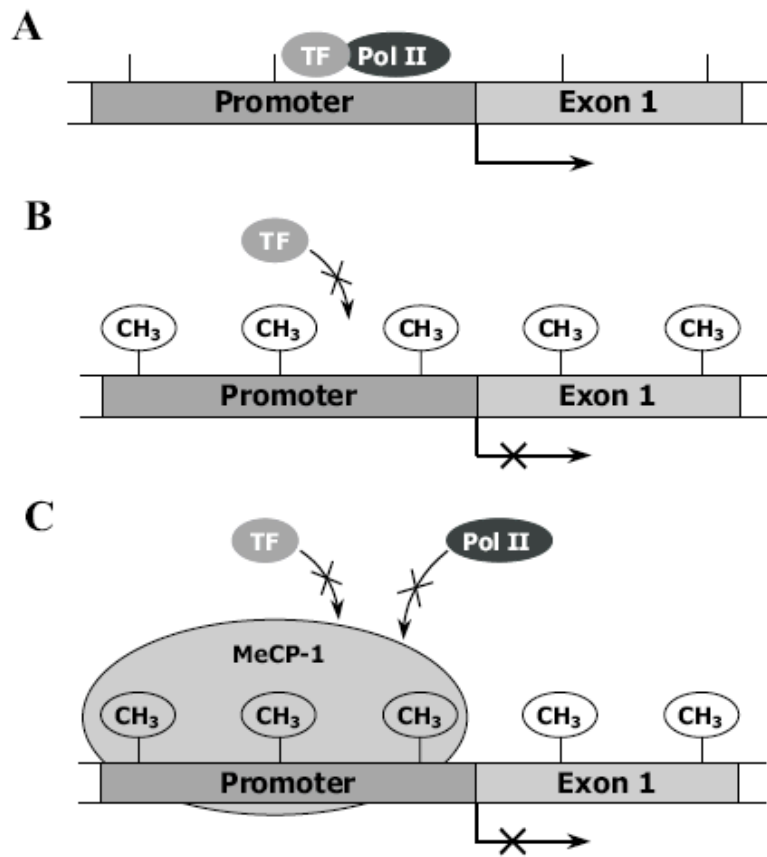
Figure 1.4. DNA methylation and inhibition of transcription (Taken from Januchowski et al., 2004; Signal and Ginder, 1999)

Abnormal DNA methylation patterns are common in most cancers, including lung, prostate, breast, and colon cancers. The DNA methyltransferase enzymes catalyze the SAM-dependent methylation of DNA, forming SAH and 5-methylcytosine. The enzyme Dnmt-1 is responsible for maintaining normal DNA methylation during cellular division (mitosis), whereas Dnmt-3a and 3b are responsible for de novo DNA methylation, an important epigenetic event (Jeltsch, 2006). In addition to global hypomethylation, which causes chromosomal instability and site-specific hypermethylation, which inactivates DNA repair and cell regulatory mechanisms,

changes in Dnmt activity are also associated with carcinogenesis (Momparler and Bovenzi, 2000). The increase in Dnmt activity may be in response to fluctuations in cellular SAM homeostasis during methyl deficiency (i.e., cofactors). In terms of DNA methylation, cancer can be described as a general loss of methyl groups at CpG islands thereby promoting the activation of normally inactivated genes that influence cell growth (Momparler and Bovenzi, 2000).

Feeding animals a diet absent of "methyl groups" (e.g., choline ad methionine) causes progressive loss of histone H3 lysine-9 trimethylation, H4 lysine-20 trimethylation, H3 lysine-9 acetylation, and H4 lysine-16 acetylation (Pogribny et al., 2007). Histones are highly conserved proteins that organize and compact DNA into structural units called nucleosomes. Histones contain a globular carboxy-terminal end that makes up its core structure and a flexible amino-terminal tail that attaches to neighboring histones, forming a larger, more complex structure (Cheung and Lau, 2005). These amino-terminal tails can be modified posttranslationally by phosphorylation, ubiquitination, ribosylation, acetylation, and methylation. Histone methyltransferase activity is downregulated during methyl-deficiency, changing not only the nature of the nucleosome but signaling within the cell including upregulation of cell cycle regulatory genes (e.g., tumor-suppressor family proteins) (Pogribny et al., 2007).

Animals fed a methyl cofactor-deficient diet display increased expression of oncogenes including c-myc and c-fos (Ross, 2003). The regulatory element of the tumor suppressor gene, p53, which regulates the cell cycle (i.e., apoptosis, senescence, and repair), upon stress (e.g., UV damage, DNA adducts) is hypomethylated in early preneoplastic nodules. However, its expression is diminished in mature tumors through

hypermethylation of its promoter region, highlighting one of many complex occurrences during metastasis (Ross, 2003).

DNA methylation status may also be an important factor in certain autoimmune diseases, namely systemic lupus erythematosus (SLE) (Januchowski et al., 2004). Autoreative T cells (Th2-CD4+) exhibit hypomethylated DNA and reduced Dnmt-1 methyltransferase activity, in turn over-expressing costimulatory molecules that propagate autoantigen formation, inflammation, and activation of other effector cells (i.e., B cells, NK cells, Th1/CD8+ cells) and their products (i.e., antibodies, complement, chemokines, and cytokines) against self-antigens (Palmer, 2003; Pan and Sawalha, 2008). The initiation of lupus pathogenesis is undefined and exposure to certain drugs, such as blood pressure medications may represent one possibility, as they can interfere with cellular signaling (e.g., MAPK/ERK) changing the activity of Dnmt(s) (Lu et al., 2005; Sawalha et al., 2008). The drug 5-azacytidine, a Dnmt-1 inhibitor, can induce autoreactivity in T cells and produce lupus-like symptoms in mice (Quddus et al., 1993). B cells exposed to high concentrations of single-stranded hypomethylated $(CpG)_n$-DNA, a highly conserved pathogen associated molecular pattern (PAMP) recognized by TLR-9, form immune complexes and stimulate the formation of autoreactive dendritic cells instead of non-reactive follicular B cells (Lenert, 2006). Therefore, environmental exposure to chemicals and pathogens represent two important disease-related epigenetic phenomena.

Genistein

A balanced diet low in lipids rich in saturated fatty acids and high in fruit, vegetable, and grain content is associated with a decreased risk of developing chronic disease including cardiovascular disease and certain cancers. In addition to vitamins, minerals and fiber, plant-based foodstuffs contain numerous bioactive compounds that also contribute largely to their associated health benefits. The isoflavanoids are a group of such constituents found at high concentrations in the diet of Asians, a population exhibiting a lower prevalence of chronic disease compared to those living in Western countries (Sakai and Kogiso, 2008).

Certain phytochemicals, namely phytoestrogens, elicit beneficial health effects in numerous animal and human studies including treatment and prevention of cardiovascular disease, cancer, hyperlipidemia, osteoporosis, and enhancement of immune function (Sakai and Kogiso, 2008). Isoflavones are the most abundant dietary source of phytoestrogens, a group of molecules similar in structure to 17β-estradiol (Figure 1.5).

The most common food sources of isoflavones are soybeans and soy-containing foodstuffs (e.g., tofu, soy milk, and soy sauce). In addition to soybean, other legumes, lentils, peas, grains, and clover contain isoflavones but at a lower concentration. Geographic agronomic variation and food processing and storage methods vary the isoflavone content of foods (Sakai and Kogiso, 2008). Daidzein (4',7-dihydroxyisoflavone) and genistein (4',5,7-trihydroxyisoflavone) represent the two most abundant forms of isoflavones found in soy. Both molecules have been studied

extensively in the context of hormone-related disease including treatment and prevention

of estrogen-responsive cancers, post-menopausal therapies, and cardiovascular

pathogenesis (Cappelletti et al., 2006).
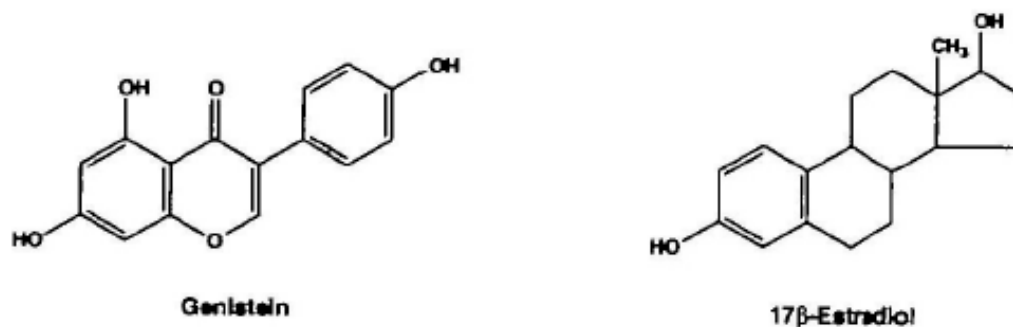
Figure 1.5. Receptor binding domain similarity of genistein and 17β-estradiol (Taken

from Wang et al., 1996)

The protective effect of estrogens in prevention of coronary artery disease is

diminished with the onset of menopause in women. However, the use of hormone

replacement therapy (HRT) to prevent bone-loss and reduce the side affects of

menopause may promote the risk of breast cancer, venous thrombosis, ovarian cancer,

and stroke. It is recommended that HRT is administered at the lowest dosage and

shortest duration required to alleviate menopausal symptoms (Marini et al., 2009).

Estrogens influence cellular growth and differentiation and function through estrogen

receptors, which are ligand-activated transcription factors (Katzenellenbogen et al.,

2000).

Genistein and Health

In 1999, the Food and Drug Administration (FDA) began to label soy products as heart healthy (Sacks et al., 2006). In 2006, the American Heart Association (AHA) determined, based on previous research findings, that both soy isoflavones and protein do not provide significant protection against cardiovascular risk factors.

Early animal studies indicated that soy protein substituted for animal protein could reduce LDL cholesterol and have beneficial effects on triacylglycerol profile. However, when applied to a vegetarian diet, no additional cholesterol-lowering effect was determined (Sacks et al., 2006). Data stemming from the original work, including complete protein replacement by soy in an omnivore diet, was confounded due to additional, unaccounted changes in food lipid profiles (Marini et al., 2009). The AHA meta-analysis argues that dietary soy isoflavones have minimal benefits on HDL-cholesterol, triacylglycerol profile, blood pressure, vasomotor menopause symptoms, and slowing osteoporosis (Sacks et al., 2006). Additionally, the estrogenic actions of genistein may pose safety concerns when applied as a treatment of breast, endometrial, and prostate cancer (Sacks et al., 2006).

In plants, isoflavones are associated with proteins as bound glycosides (conjugated glycones). Intestinal microflora can ferment these compounds, removing the associated sugar residue, forming the aglycones genistein and daidzein from genistin and daizdin, the most abundant forms of isoflavones in soy products. Free genistein and daidzein are absorbed across the intestinal lumen epithelium efficiently as well as in the stomach (Marinin et al., 2009). In the colon, bacteria can further metabolize daidzein to equol and O-demethylangolesin and genistein to p-ethyl phenol. Once absorbed, these

compounds are not subject to further metabolism and represent collectively the most abundant circulating and excreted forms of isoflavanoids. Typically, 250-350 mg/kg diet genistein is administered to rodents to model intake by individuals regularly consuming soy foodstuffs without supplementation. Such a diet, common in Asian and Western vegetarian populations, contains 1.4 mg/kg body weight genistein (Coward et al. 1993). Infants consuming soy-based formulas can achieve intakes over 5 mg/kg body weight genistein daily (Setchell, et al., 1997). Women who regularly consume soy foodstuffs exhibit plasma genistein concentrations of 0.7-6.0 µmol/L (Fang et al., 2007).

Genistein and Estrogens

The structural similarity of genistein to 17β-estradiol suggests its ability to mediate cellular processes in a hormone-like fashion. Endogenous estrogens can regulate transcriptional activation and repression of both estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β) (Cappelletti et al., 2006). These receptors differ in the amino acid profiles of their N-terminal domains (82%) and hormone-binding domains (44%) (Katzenellenbogen et al., 2000). These differences explain in part the varying affinity of ligands between receptor subtypes, and the paradoxical (e.g., agonist versus antagonist) effects of binding ligands (Katzenellenbogen et al., 2000). Despite its similar structure, the binding affinity of genistein to ER-α is 0.5 to 1% of that of 17β-estradiol (Ye et al., 2009). However, genistein binds ER-β at even higher affinity than 17β-estradiol, functioning as a selective estrogen receptor modulator (SERM) (Ye et al., 2009). In this role, genistein selectively serves as both an estrogen receptor agonist and antagonist depending upon the tissue of interest and dosage (Dolinoy et al., 2006; Price and Fenwick, 1985).

Estrogen and Disease

The atheroprotective effect of estradiol is mediated through endothelial ER-α by cyclooxygenase-2, direct inhibition of lipid deposition on endothelial cells, rather than changes in blood lipid profile (Billon-Gales et al., 2009). In addition, estradiol may mediate the adaptive immune response away from the production of proinflammatory cytokines (Elhage et al., 2005). Conversely, the protective role of genistein in estrogen-responsive cancers is believed to be consequence of its structural binding to ER-β. Both estrogen dependent and independent mechanisms have been suggested for the positive and negative immune modulatory effects of genistein. Inhibition of estrogen signaling pathways, in lymphocytes and macrophages, reduces the effect of genistein on these cells (Sakai and Kogiso, 2008). Upon binding to ER-β, genistein recruits coregulatory proteins to the receptor and forms an AF-2 helix, which inhibits estrogen agonist activity and thus represents its antagonist regulation (Cappelletti et al., 2006). Cappelletti et al. (2006) determined that genistein, through the action of ER-β, inhibits estrogen-related cell growth but does not inhibit estrogen-regulated transcription (Cappelletti et al., 2006).

Genistein also has been shown to interact with other steroid nuclear receptors including the vitamin D receptor (VDR). Genistein can upregulate both the expression and abundance of VDR in estrogen-positive breast cancer cell lines (Wietzke and Welsh et al., 2003). The tissue-specific molecular actions of genistein are not completely understood. Compared with 17β-estradiol, genistein exhibits paradoxical effects of other synthetic estrogen molecules developed for health interventions.

The structure of genistein and daidzein is also similar to the synthetic estrogens diethylstilbestrol (DES) and hexestrol, respectively, altering the same 179 genes in the

immature mouse uterus causing a parallel uterotrophic response (Moggs et al., 2004).

These potent non-steroidal anabolic compounds have been used widely to regulate sexual

maturation, growth in livestock, and for treatment of estrogen-responsive diseases (e.g.,

uterine fibromyoma and sterility). However, since their discovery in the first half of the

20th century, safety concerns have limited their widespread application (Verloop and

Leeuwen, 2010). Women prescribed DES between 1938 and 1971 during pregnancy for

miscarriage prevention are 30% more likely to develop breast cancer compared to the

normal population. DES "sons" are at increased risk to develop non-cancerous

epididymal cysts and "daughters" bear the greatest detriment, including clear cell

adenocarcinoma, reproductive tract defects (e.g., T-shaped uterus), and pregnancy

complications including ectopic implantation, preterm delivery, and sterility. Recent

literature has indicated that third generation DES individuals may also be at risk for

reproductive defects (Titus-Ernstoff et al., 2008).

Aromatase (CYP19), a cytochrome P450 enzyme, catalyzes the final rate-limiting

step in the synthesis of estrogen from cholesterol. In the endoplasmic reticulum,

aromatase performs three hydroxylation reactions of the 19-methyl group of androgen

molecules derived from cholesterol, removing the methyl-group as formate and

transforming the left-hand A-ring structure to an aromatic state. Exogenous estrogen

administration or perturbations in endogenous estrogen synthesis, including

polymorphisms in the CYP19 gene have been shown to induce and accelerate mammary

carcinogenesis in mice (Yoshidome et al., 2000).

Estrogen promotes cellular proliferation via signaling and involvement in cell

cycle regulation, including modulation of various proteins and transcription factors

related to growth and apoptosis. Ye and colleagues (2009) determined that treatment with 10 μmol/L genistein was able to increase aromatase activity and expression in HepG2 cells through phosphorylation of the protein kinases (PKCs), extracellular signaling kinase (ERK), p38, and mitogen activated protein kinase (MAPK), in turn promoting the DNA binding of CREB to CYP19. Perturbations in estrogen homeostasis mediated by aromatase are associated with the development of hepatocellular carcinoma (HCC), indicating that genistein administered at significant quantities may promote the survival and growth of estrogen responsive neoplastic cells.

Genistein and Disease

An achievable, single intake of genistein (30-60 mg) produces a blood concentration of roughly 1 μmol/L, assuming there is minimal microbial degradation in the small intestine and variable absorption of 13-46% based on luminal perfusion data and human research accounting for variation in gut-flora (Andlauer et al.2000). Circulating concentrations of genistein exceeding 4 μmol/L can produce estrogenic effects in laboratory animals (Penza et al., 2007). Genistein concentrations in the blood drop significantly within 12-24 hours after ingestion and are found to be localized largely in the liver and reproductive tissues, including the uterus, testes, vagina, prostate, and prostatic fluid (Penza et al., 2007). Genistein metabolism and hepatic biotransformation is similar between rats and humans, which form glucuronide conjugates upon metabolism. The estrogenicity of the glucuronide conjugates is less potent than that of genistein. However, the activity of these products is not completely understood (Bursztyka et al., 2008).

Genistein also functions broadly as a tyrosine-kinase inhibitor of cellular growth factors including pathways of epithelial growth factor receptor (EGFR) and the oncogenes pp60v-src and pp110gag (Akiyama et al., 1987). Regulation of the EGFR is mediated through competitive binding in multiple places of the reaction pathway and not solely as a competitive binder of phosphate (e.g., ATP-binding sites) (Akiyama et al., 1987). Genistein has been shown to inhibit the growth of estrogen-responsive LNCaP by inducing G_0-G_1 phase arrest through upregulation of the tumor suppressor gene, cyclin p21. Genistein also inhibits the G_2-M phase of cellular replication in breast, gastric, lung, and human melanoma cells lines through the action of CDK inhibitors p21WAFI and cyclin B1, which govern DNA replication (Majid et al., 2008).

Genistein can induce apoptosis in cancer cells by upregulating BRCA1 and the tumor suppressor gene p16. The promoter regions of these genes are normally hypomethylated in cancer cell lines. Genistein treatment does not methylate the CpG promoter regions of these regulatory genes. Therefore, the anti-cancer activity of genistein may not be related to changes in the DNA-methylation patterns. However, genistein was found to increase histone (H3 and H4) acetylation in a dose-response fashion near the transcription site of the regulatory genes. Genistein was found to upregulates histone acetyltransferases (HAT) and induces chromatin remodeling, which in turn may represent a component of its anti-cancer activity (Majid et al., 2008). However, genistein has been shown to alter prostate DNA methylation patterns in mice but not in liver tissue, regardless if the animals were fed control diet weeks before or after genistein supplementation, indicating that changes in estrogen-responsive tissue DNA methylation may be stably altered (Day et al., 2002). In this regard, genistein has been

shown to reduce the risk of cancer in female rats whose mothers were fed genistein. However, post-natal supplementation did not provide a protective effect (Warri et al., 2008).

The proposed anti-atherogenic activity of genistein is similar to that of endogenous estradiol in that it may lower plasma homocysteine concentrations, prevent LDL oxidation, improve vascular tone, and decrease the production of inflammatory cytokines, reactive nitrogen species and cell-adhesion molecules (Fuchs et al., 2005). Genistein treatment (2.5 μmol/L) has been shown to prevent homocysteine-induced (25 μmol/L) cytoskeleton protein changes in endothelial cells, inhibiting apoptosis, which was determined by measurement of DNA-fragmentation and chromatin condensation (Fuchs et al., 2005).

Genistein has been shown to decrease the activity of all three DNA methyltransferase (e.g., Dnmt-1, 3a, and 3b) in both benign and malignant breast cancer cells, down-regulating telomerase activity by inhibiting the transcription of the human telomerase reverse transcriptase promoter (hTERT), causing cellular arrest and apoptosis (Li et al., 2009). Genistein has been shown to induce permanent alterations in the epigenome by using the agouti mouse model. Administration of genistein at 250 mg/kg diet to pregnant female agouti mice shifted the offspring's coat color toward pseudo-agouti and prevented adiposity. Genistein was found to hypermethylate CpG sites upstream of the of the promoter region of the agouti gene, reducing yellow phaeomelanin production and protecting against adult-onset adiposity (Dolinoy et al., 2006). These changes persisted into adulthood and the alterations in DNA methylation were independent of one-carbon metabolism (Dolinoy et al., 2006). These results demonstrate

the effects of environmental factors on epigenetic regulation during prenatal and early

postnatal development and also suggest that folate status is very important when

consuming genistein, as potentially additive effects may occur in relation to DNA

methylation altering cellular function and ultimate disease susceptibility.

CHAPTER 2. GENISTEIN MEDIATES PERTURBATIONS IN ONE-CARBON METABOLISM DURING DIET-INDUCED FOLATE DEFICIENCY

Abstract

Populations consuming isoflavone-rich diets exhibit lower prevalence of heart disease and estrogen-responsive cancers. Genistein, a17β-estradiol-like isoflavone, has been shown to modify the fetal epigenome in utero in the agouti mouse model and modulate DNA methylation patterns in estrogen-responsive tissues. Genistein also exhibits antiproliferative action in cancerous cells via suppression of S-adenosylmethionine-dependent DNA methyltransferases, leading to site-specific hypomethylation in cell cycle regulatory genes. However, it is not known if genistein modulates one-carbon metabolism, a process that when disrupted leads to dysregulation of the epigenome. The objective of these studies was to characterize the metabolic actions of genistein related to one-carbon metabolism, including the ability of genistein to reduce plasma homocysteine, and alter transmethylation, remethylation, and transsulfuration enzymes perturbed by folate deficiency. Male Sprague Dawley rats were fed either a folate adequate (Ctrl) or deficient diet (FD) with or without genistein (300 mg/kg diet). In the first study, rats were fed an FD diet for a total of 38 d, and were administered either genistein (300 mg/kg diet) or creatine monohydrate (4 g/kg diet) during the last 10 d of the study period. In the second study, rats were administered genistein for 24 of the 38 total d. In the third study, rats were acclimated for 5 d on a Ctrl diet prior to feeding a FD diet for 59 d with simultaneous genistein administration. Moderate folate deficiency did not alter enzyme function or genomic DNA methylation

patterns. However, the associated hyperhomocysteinemia was attenuated in all three studies by genistein. Genistein administration had no effect on plasma homocysteine concentrations in Ctrl fed rats. Genistein administration was not found to alter the activity or expression of hepatic enzymes regulating one-carbon metabolism or produce alterations in genomic DNA methylation patterns. Genistein may be acting on estrogen-responsive tissues, altering homocysteine metabolism. Genistein could also be mediating one-carbon metabolism independently of folate via an unknown mechanism. Future research is required to better understand the actions of genistein related to its ability to modulate homocysteine metabolism.

Introduction

Hyperhomocysteinemia is associated with various pathological conditions including cardiovascular disease, neural tube defects, neurodegenerative diseases, and cancer (Cattaneo, 1999). Homocysteine, a non-protein amino acid, is an intermediate in methyl group metabolism. S-adenosylmethionine (SAM), the major methyl group donor for all organisms, forms S-adenosylhomocysteine and ultimately homocysteine. Upon formation, Homocysteine undergoes remethylation to generate methionine by both folate-dependent and independent routes or undergoes catabolism to cysteine via the transsulfuration pathway (Figure 2.1) (Wagner, 1995). Reduced intake of folic acid, B_{12}, and pyridoxine (B_6) contribute to elevated plasma Homocysteine, perturb cellular methyl potential, and represent biomarkers for epigenetic perturbations (Fux et al., 2005). In addition, missense mutations in the 5,10- methylenetetrahydrofolate reductase (MTHFR) gene (C677-position polymorphism) causes thermolability and attenuated activity of MTHFR, an enzyme responsible for the formation of 5-methyl-THF, which remethylates

Homocysteine back to methionine via methionine synthase (MS) (Ilhan et al., 2008).

Polymorphism in genes regulating folate metabolism, such as MTHFR, exist in upwards of 15% of the population and pose substantial risk for certain individuals, namely women of child-bearing age (Bailey and Gregory, 1999). The SAM-dependent enzymes guanidinoacetate N-methyltransferase (GAMT) and phosphatidylethanolamine N-methyltransferase (PEMT) form creatine and phosphatidylcholine respectively, consuming upwards of 80% of the tightly regulated methyl groups (Figure 2.1). Research conducted by Brosnan and colleagues has determined that supplementation with creatine monohydrate (4 g/kg diet) significantly reduces plasma homocysteine concentrations by 50%, while administration of its precursor, guanidinoacetate, inversely increased homocysteine concentrations by 25% (Stead et al., 2001). The remaining SAM-derived methyl donations are allocated largely to DNA methylation, an epigenetic process influenced by the dietary isoflavone genistein (Day et al., 2002). DNA methyltransferase (DNMT) 1, 3a, and 3b, which govern maintenance and de novo DNA methylation, respectively, are potential targets for cancer therapy.

Epigenetic regulation includes cellular changes that modify transcription, translation, and posttranslational events without alterations of the underlying base sequence of DNA. Recently, epigenetic regulatory mechanisms, including DNA methylation, have become increasingly implicated in various diseases (Oommen et al., 2005). Genistein (4', 5,7-trihydroxyisoflavone), a 17β-estradiol-like isoflavone, has been shown to maintain normal methyl group metabolism in the agouti mouse model fed a methyl-deficient diet. Maternal genistein supplementation during gestation produces pseudo-agouti offspring via hypermethylation and suppression of regions upstream of the

agouti gene (Dolinoy et al., 2006). In the human diet, genistein is largely consumed via soy-based foodstuffs or supplements used as therapy for menopausal. Although dietary genistein does not directly provide a methyl group for metabolism, it may modulate the one-carbon pool and influence DNA methylation particularly in estrogen-responsive tissues (Fang et al., 2007). Genistein, as a dietary agent, may have the greatest effect on cellular events during embryonic development (gestation), puberty, and lactation, when hormones such as estrogens influence maturation (Cappelletti et al., 2006).

Genistein interacts with a number of cellular receptors, including the estrogen β-receptor, which it binds at an even higher affinity than endogenous 17β-estradiol, resulting in both activating and inhibitory regulation within the cell (Cappelletti et al., 2006). Recently, genistein was shown to down-regulate telomerase activity in cancer cells, inducing apoptosis. Genistein can induce repression of telomerase through hypomethylation of the hTERT promoter in a similar fashion to the DNMT-1 inhibitor 5-2'-deoxycytidine in breast cancer cells (Li et al., 2009). The extent genistein directly influences methyl group and SAM/Homocysteine metabolism is not known (Figure 2.1).

In the present study, the effects of supplemental genistein on elevated plasma Homocysteine, during diet-induced, moderate folate deficiency were examined. The supplement dose was chosen to mimic intakes by individuals consuming a diet rich in soy-based products (e.g., tofu and soy milk). By using a folate restriction model, our study aimed to represent portions of the population with reduced folate absorption, intake, or altered metabolism (e.g., geriatric populations or MTHFR variants) (Wilcken and Wilcken, 1998). Previous research investigating the effect of genistein on Homocysteine metabolism is limited to the study of its role as an antioxidant, although

there are reports regarding its use in the prevention of Homocysteine-related pathologies, including cardiovascular disease and cancer (Fuchs et al., 2005). By examining the specific interaction of genistein in one-carbon metabolism in vivo we can begin to understand its global actions during chronic disease.

Experimental Procedures

Animals and Diets

Experimental Design 1

Animal care and experiments were conducted in accordance with the Iowa State University, Institutional Animal Care and Use Committee (IACUC), Laboratory Resource Guidelines. Male Sprague Dawley rats (Harlan Teklad, Sprague Dawley, Indianapolis, IN) were assigned randomly to individual plastic cages and placed in a room with a 12-h light:dark cycle. Rats were allowed access to food and water ad lib. Rats were obtained at 3-4 weeks of age (50-74g) and fed for 28 d on an AIN-93 (Harlan Teklad, Indianapolis, IN) control diet containing, 10% vitamin free casein, 0.3% L-methionine, 40.2% corn starch, 39.3 % dextrose, 5% corn oil, 4% mineral mix (AIN-93G-MX), 1% vitamin mix (AIN-93-VX), and 2% choline bitartrate (1.5 mol/L) to promote growth. Rats were assigned to 6 groups (6 rats per group) fed the following diets: control (2 ppm folate), deficient (0 ppm folate) during the 28-d period. Parallel diets supplemented with 300mg/kg diet 99%+ purity genistein (Indofine Chemical Company, Hillsborough, NJ; G-103) or 4 g/kg diet creatine monohydrate (Sigma Aldrich, St Louis, MO) were fed for an additional 10 d. Cornstarch (0.3 g and 4.0 g) was removed from the diet and replaced with genistein or creatine.

Experimental Design 2

Male Sprague Dawley rats (Harlan Teklad, Sprague Dawley, Indianapolis, IN) were assigned randomly to individual plastic cages and placed in a room with a 12-h light:dark cycle. Rats were allowed access to food and water ad lib. Rats were obtained at 3-4 wk of age (50-74g) and placed in a diet containing, 10% vitamin free casein, 0.3% L-methionine, 40.2% corn starch, 39.3 % dextrose, 5% corn oil, 4% mineral mix (AIN-93G-MX), 1% vitamin mix (AIN-93-VX), and 2% choline bitartrate (1.5 mol/L) with either adequate folate (2 ppm folate) or folate-deficient vitamin mix (0 ppm folate) for 14 d prior to supplementation with 300 mg/kg diet 99%+ purity genistein (Indofine Chemical Company, Hillsborough, NJ) for an additional 24 d. Cornstarch (0.3 g) was removed from the diet and replaced with genistein.

Experimental Design 3

Male Sprague Dawley rats (Harlan Teklad, Sprague Dawley, Indianapolis, IN) were assigned randomly to individual plastic cages and placed in a room with a 12-h light:dark cycle. Rats were allowed access to food and water ad lib. Rats were obtained at 3-4 wk of age (50-74g) and acclimated for 5 d on an AIN-93 (Harlan Teklad, Indianapolis, IN) control diet containing, 10% vitamin free casein, 0.3% L-methionine, 40.2% corn starch, 39.3 % dextrose, 5% corn oil, 4% mineral mix (AIN-93G-MX), 1% vitamin mix (AIN-93-VX), and 2% choline bitartrate (1.5 mol/L) to promote growth. Rats were randomly assigned to 4 groups (6 rats per group) fed the following diets: control (2 ppm folate), deficient (0 ppm folate), and parallel diets supplemented with 300 mg/kg diet 99%+ purity genistein (Indofine Chemical Company, Hillsborough, NJ) for an

additional 59 d. Cornstarch (0.3 g) was removed from the diet and replaced with genistein.

At necropsy, non-fasted rats were anesthetized at 5 hr into the light cycle with a single intraperitoneal injection of fresh ketamine (135 mg/kg body weight) and zylazine (15 mg/kg body weight) mixture. This ratio was adjusted from 90 mg/kg body weight ketamine and 10 mg/kg body weight zylazine to prevent additional injections, as individual rats respond differently to anesthesia. Heparinized whole blood samples were collected by cardiac puncture and centrifuged at 4000 x g for 6 min upon separation of plasma fraction. Plasma samples were frozen at -20°C for subsequent analysis of homocysteine concentrations by high performance liquid chromatography (HPLC).

One-gram portions of liver were rapidly removed and placed into tubes containing 4 ml of ice-cold buffer comprised of 10 mM sodium phosphate (pH 7.0), 1 mM EDTA, 0.25 M sucrose, 1 mM sodium azide, and 0.1 M phenylmethylsulfonyl fluoride. The mixture was homogenized and centrifuged at 20,000 x g at 4°C for 30 min. The protein portion was transferred to 1.5 ml eppendorf tubes and stored at -80°C for later enzyme analysis. The remainder of the liver was removed and placed immediately in liquid nitrogen and stored at -80°C for later analysis, including genomic DNA and RNA isolation. One kidney was removed from each animal and homogenized in 1:4 wt/vol homogenate buffer as described above and centrifuged at 20,000 x g at 4°C for 30 min. β-mercaptoethanol was added to each liver and kidney protein fraction at a final concentration of 1 mM. The samples were stored at -80°C for later enzyme analysis.

Bicinchoninic Acid Protein (BCA) Concentration

Isolated proteinaceous fractions from liver and kidney homogenates were thawed on ice and analyzed to determine protein concentration by bicinchoninic acid protein (BCA) method (Thermo Scientific, Rockford IL; Pierce Microplate BCA Protein Assay Kit). To adjust for an appropriate standard curve, 2.5 μl instead of 5 μl of protein sample was added to each well in triplicate. Liver and kidney protein isolates were found to be more concentrated than protein derived from cell culture, as described by the original protocol. Samples were pippeted onto a 96-well plate in triplicate (2.5 μl protein). Bovine serum albumin (BSA) was used to establish a standard curve. The prepared standard curve was established by plotting the average blank-corrected value versus its concentration (μg/ml) in ascending order from 100% deionized H_2O to 100% BSA. In addition to protein, 12.5 μl of deionized H_2O was added to each well. Approximately 160 μl of Pierce BCA reagent A and B were added rapidly to each well, including the wells containing BSA. Reagent A (stock mixture = 250 μl) contains, sodium bicarbonate, sodium carbonate, bicinchoninic acid, and sodium tartarate dissolved in 0.1 M sodium hydroxide. Reagent B (stock mixture = 25 ml) contains a 4% cupric sulfate solution. Prior to absorbance at 562 nm, the 96-well plate was covered and allowed to sit at room temperature for approximately 45 min.

High Performance Liquid Chromatography

Plasma samples were thawed and total plasma homocysteine concentrations were determined using methods originally described by (Ubbink et al., 1991). Approximately 300 μl of plasma from each sample was spiked with 1 mM N-acetylcysteine and incubated at 4°C for 30 min in 1:10 vol/vol of tributylphosphine in dimethylformamide. The reaction was terminated by adding 1 mM EDTA dissolved in 10% trichloroacetic

acid. The reaction mixture was centrifuged at 4°C for 5 min at 1000 x g. The collected supernatant was added to tubes containing 0.125 borate buffer (pH 9.5), 1.55 M sodium hydroxide, and 0.1% 4-flouro-7-sulfobenzofurazan. Each sample was filtered using aerodisc filters (0.22 μm) prior to injection (100 μl) into the HPLC. HPLC preparation: Samples were analyzed under fluorescence detection using a C-18 column. The HPLC solutions included 0.1 M KH_2PO_4 buffer (pH 2.1), 100% acetonitrile, and deionized water. During the running period, the solutions were adjusted to 96% sodium phosphate and 4% acetonitrile. The flow rate was set at 2.0 ml/min, and pressure was maintained at 1000-1500 psi. Chromatograph data indicated that the homocysteine peak occurred at approximately 6 min and the N-acetylcysteine peak at approximately 20 min.

Glycine N-methyltransferase (GNMT) Activity

Glycine N-methyltransferase (GNMT) activity was determined by a method described by Cook and Wagner. The experiment was performed on both liver and kidney samples. The reaction mixture included 200 mM Tris buffer (pH 9.0), 2 mM glycine, 0.2 mM S-adenosyl-L-[methyl-^{3}H]methionine, and 5 mM dithiothreitol. The S-adenosyl-L-[methyl-^{3}H]methionine was made by adding 23 μl (SAM^{3}H) to (10 mM SAM) and diluting at 1:10 vol/vol with 10 mM H_2SO_4. Protein aliquots from each sample, adjusted to include 250 μg of protein, were added to separate eppendorf tubes and denatured at 80°C for 15 min for background analysis. Previously, it was recommended to add 45 μl of protein to each tube, denature, and then add an appropriate aliquot to each reaction mix. However, isolating the exact amount of protein was found to be difficult because the denaturing process dehydrates the sample. The reaction was performed in triplicate with one blank. The reaction was initiated by adding each sample to the reaction mix and

incubating at 25 °C for 30 min. The reaction was terminated by the addition of 50 µl of 10% trichloroacetic acid. Upon termination, 250 µl of activated charcoal suspension (78 mg/ml in 0.1 M acetic acid) was added to each sample and the resulting solution was placed in a 4°C refrigerator for 20 min prior to centrifugation at 4°C for 5 min at 13,500 x g. Approximately 200 µl of supernatant from each sample was added to scintillation vials containing 10 ml Scintiverse (Fisher Scientific, Fair Lawn, NJ) to determine radioactivity by liquid scintillation counting.

Methionine Synthase (MS) Activity

Methionine synthase activity was determined by a method originally described by Keating and colleagues. Resin preparation: 25 g of AG 1-X8 resin (Cl$^-$ form, 100-200 mesh) was suspended in a 1 L beaker and washed 3 times with 300 ml deionized H_2O to remove impurities. To prime the resin, 300 ml 0.5 M HCl was added and allowed to rest for 90 min, followed by 3 additional rinses with 300 ml deionized H_2O. Next, 300 ml of 0.5 M sodium hydroxide was added to the resin and rinsed 3 times with 300 ml deionized H_2O. Finally, 500 ml of 0.5 M HCl (counterion) was added and decanted and the resin was rinsed with 100 ml deionized H_2O to remove excess ions. The resin was vacuum filtered (Whatman filter paper) using a Buchner funnel, washed, and stored in deionized H_2O at room temperature. Reagents: The reaction mix included the following: freshly made 100 mM DL-homocysteine, 1.3 mM cyanocobalamin, 1 M dithiothreitol, 10 mM S-adenosylmethionine, 82.4 mM 2-mercaptoethanol, 500 mM sodium phosphate buffer (pH 7.5), 5-CH$_3$-tetrahydrofolate, 5-[^{14}CH$_3$]-tetrahydrofolate (0.25 µCi), and deionized H_2O. Hepatic protein samples from each rat were diluted with deionized H_2O to 600 µg in 100 µl. Two samples were denatured at 75°C for 10 min and used as blanks without 100 mM

DL-homocysteine. Each sample (100 μl) was spiked with 100 μl of reaction mix and incubated at 37 °C for 1 hour. The reaction was terminated by the addition of 800 μl of ice-cold water, and each sample was added to columns containing approximately 1 ml of resin. The sample was rinsed twice with 1 ml deionized H_2O. Scintillation vials were labeled A, B, and C for each sample, and effluent was collected as it passed through the resin. The scintillation vials were filled with 10 ml Scintiverse for radioactivity determination by liquid scintillation counting.

Phosphatidylethanolamine N-methyltransferase (PEMT) Activity

Approximately 1 gram of liver was isolated from the total liver sample and homogenized in 4 ml of 10 mM Tris-HCl (pH 7.4) and 0.25 M sucrose (PEMT buffer). The homogenate was centrifuged at 16,000 x g for 20 min at 4°C. The proteinaceous supernatant (1-2 ml) was collected and ultracentrifuged for 1 hour at 105,000 x g at 4°C (TLA 100.4 rotor at 50,000 rpm). The resulting supernatant was discarded and the microsomal pellet was resuspended by gentle vortex in approximately 0.5 ml 0.25 M sucrose. Protein concentration was determined by BCA method, and the reaction mixture was adjusted to include 750 μg protein in a final volume of 475 μl. The reaction mixture included the following: 10 mM HEPES (pH 7.3), 5 mM $MgCl_2$, 4 mM dithiothreitol (DTT), 0.1 mM SAM, 2 μCi S-adenosyl-L-[methyl-3H]methionine, and 750 μg protein. The reaction was initiated by adding 75 μl of microsomal protein to the reaction mixture (475 μl) and incubated at 37 °C for 10 min. To terminate the reaction, 100 μl aliquots were added in duplicate to closed-glass tubes containing 2 ml of chloroform (1.2 ml), methanol (600 μl), and 2 N HCl (200 μl) respectively. The chloroform phase was washed three times with 1 ml 0.5 M KCl dissolved in 50% methanol. To maintain the

chloroform phase after washing samples were centrifuged at 4 °C for 5 min at 1800 x g. The remaining chloroform phase was transferred to a glass scintillation vial, and the glass washing tube was rinsed three additional times with chloroform to remove any remaining lipid fraction. The chloroform was evaporated overnight, and 5 ml Scintiverse was added for radioactivity determination by liquid scintillation counting.

DNA Isolation and Global Methylation Analysis

The method described by Pogribny and colleagues was used to determine relative hepatic DNA methylation. Isolated DNA from each sample was digested with a methyl-sensitive restriction enzyme exhibiting endonuclease activity for CpG dinucleotides. After enzymatic cleavage, the cytosine extension assay was used to determine ^{3}H incorporation into staggered DNA fragments.

DNA isolation (Promega Wizard Genomic DNA Purification Kit, Madison, WI): 600 µl of Nuclei Lysis Solution was added to a 15 ml tube containing 10-20 mg of freshly thawed liver tissue and homogenized. The lysate was added to a 1.5 ml tube and incubated for 20 min at 65 °C at which point 3 µl of RNase solution was added and gently mixed. Upon reaching room temperature, 200 µl of Protein Precipitation Solution was added to the solution, vortexed, and cooled on ice. Each sample was centrifuged for 4 min at 13,000-16,000 x g and the supernatant, containing the DNA, was transferred to a new 1.5 ml eppendorf tube containing 600 µl of 70% ethanol and gently inverted until DNA strands were visible to the eye. The samples were centrifuged for one min at 13,000-16,000 x g at 4 °C, the supernatant was removed, and the tubes were inverted on bench paper to dry for 15 min. DNA Rehydration Solution (100 µl) was added the DNA and incubated overnight at 4 °C. DNA purity and concentration for each sample was

determined by NanoDrop (Thermo Scientific) method, and the DNA methylation assay was adjusted accordingly for each sample (H_2O of the reaction mix). Global methylation: DNA digestion was performed in triplicate with one blank per sample containing no endonuclease. Each eppendorf tube contained 1.25 μg DNA, 1.25 μl (12.5 U) of HpaII endonuclease (5'…C⇓CGG…3': 3'…GGC⇓C…5'), 2.5 μl 10X NEBuffer 1 (10 mM Bis-Tris-Propane-HCL, 10 mM $MgCl_2$, and 1 mM Dithiothreitol) and deionized H_2O for a total volume of 25 μl. The tubes were placed in open incubation racks and incubated for approximately 16 hr at 37 °C. Each sample was placed on ice to terminate the reaction. Cytosine extension: Briefly, each eppendorf tube contained 20 μl of digested or control DNA (1.0 μg; 20 μl), 10X PCR Buffer II w/o $MgCl_2$ (4.0 μl), 25 mM MgCl (1.6 μl), AmpliTaq DNA Polymerase (0.16 μl), [3H]-dCTP (42.9 Ci/mmol, NEN Life Science Products; 0.16 μl), and 14.08 μl deionized H_2O. To prevent evaporation, mineral oil (25 μl) was added each sample and incubated at 55 °C for 1 hour. A one in^2 grid was overlaid on DE81 anion exchange filter paper (Whatman, Piscataway, NJ; 3658-917) and 30 μl of reaction product was pipetted onto the center of each square and allowed to dry for approximately 1 hour. The filter paper wash washed 3 times for 10 min with 250 ml, 0.5 M sodium phosphate buffer to remove excess ^3H (unbound). The paper was air dried for approximately 3 hr, the grid was cut, and the individual squares were added to scintillation vials containing 5 ml Scintiverse for radioactivity determination by liquid scintillation counting.

Quantitative Real Time PCR (qRT-PCR)

RNA isolation: Approximately 50-100 mg of liver was isolated and homogenized in liquid nitrogen using a mortar and pistol. The crushed liver was immediately

transferred to a 1.5 ml eppendorf tube containing 1 ml of Trizol Reagent (Invitrogen, Carlsbad, CA). The isolate was vortexed and centrifuged at 12,000 x g for 10 min at 4 °C t remove insoluble material. The supernatant was transferred to a new eppendorf tube and incubated at 25 °C for 5 min to dissociate nuclease proteins. Chloroform (200 μl) was added to each tube, shaken, incubated for 3 min at room 25 °C, and centrifuged at 12,000 x g for 15 min at 4 °C. The phenol-chloroform and interphase were discarded, and the clear supernatant was transferred to a clean eppendorf tube. Isopropyl alcohol was added to the sample at 1:2 vol/vol of initial Trizol reagent to precipitate the RNA, incubated at 25 °C for 10 min, and centrifuged at 12,000 x g for 10 min at 4 °C. The supernatant was discarded, and the RNA pellet was washed with molecular grade 75% ethanol (1 ml) 2 times, and stored overnight in molecular grade 100% ethanol at -20 °C to remove phenol-contaminants, as determined by NanoDrop (A260/280) method. The ethanol/RNA solution was vortexed and centrifuged at 7500 x g for 5 min at 4 °C, the supernatant decanted, and air dried for 5-10 min prior to dissolving in 50 μl of RNase-free water (Ambion, Austin, TX) and incubating at 55-60 °C for 10 min. The samples were transferred to 0.5 ml eppendorf tubes containing 10X Turbo DNase-free digestion buffer and Turbo DNase I and incubated for 30 min at 37 °C (Turbo DNA-free Kit, Ambion). DNase Inactivation Reagent (beads) were added, briefly incubated at 25 °C, and centrifuged for 2 min at 10,000 x g. The RNA-contained supernatant was removed and placed in a new 0.5 ml eppendorf tube and stored at -80 °C.

Reverse transcriptase (cDNA synthesis): RNA concentrations were determined by NanoDrop method to ensure purity. Approximately 1-2 ug/ul RNA from each sample

was added to 0.2 ml eppendorf tubes containing 5X iScript reaction mix (Bio-Rad, Hercules, CA) Oligo (dt)$_{20}$ primer, iScript reverse transcriptase, and nuclease-free H$_2$O and incubated for 5 min at 25° C, 30 min at 42° C, and 5 min at 85° C. The cDNA was stored at -80° C. Polymerase chain reaction (PCR): cDNA from each sample was diluted to approximately 125 ng/μl and 1 μl was added in triplicate to a 96-well plate containing 10 pmol forward and reverse primers for the individual gene, IQ SYPR Green Supermix (Bio-Rad), and diethylpyrocarbonate (DEPC) water. The completed plate was run using the following procedure (MyIQ, Bio-Rad): 3 min at 95° C, 40 cycles for 15 s at 95° C, 30 s at 60° C, 30 s at 72° C, 1 min at 95° C, 1 min at 55° C, and 80 cycles for 15 s at 55° C. The data was analyzed using the Comparative C$_T$ method (Table 2.1) (Schmittgen and Livak, 2008).

Western Blot Analysis (Hepatic GNMT and CBS)

The protein abundance of GNMT and CBS was determined by immunoblotting methods previously described (Rowling and Schalinske, 2001). Gel preparation: Hepatic protein samples were added to eppendorf tubes containing deionized H$_2$O and 2X Laemmli Buffer diluted from 4X Laemmli (250 mM Tris HCl, 40% glycerol, 5% SDS, 0.005% bromophenol blue) and denatured for 15 min at 75 °C. The separating 15% SDS-polyacrylamide gel was comprised of 1.875 M Tris HCl (pH 8.8), deionized H$_2$O, stock acrylamide (30%), SDS (10%), APS (10%), and Temed. After polymerization, the stacking gel (0.6 M Tris HCl; pH 6.8) was loaded, and the gel was run overnight at 7 – 10 mA using eletrophoresis buffer (250 mM Tris, 192 mM glycine, and 0.1% SDS). The gel casts were separated and the polyacrylamide gels were cut to include the 32-kDa GNMT and 63-kDa CBS monomer subunits. Cassette preparation: The buffer was made up of

30% transfer buffer (125 mM Tris and 960 mM glycine), 15% methanol, and 55%

deionized H_2O. The cassette was arranged in the following order: cathode (-), 2 pieces of

Whatman filter paper, SDS-polyacrylamide gel, nitrocellulose membrane, 2 pieces of

Whatman filter paper, and cathode (+). The transfer was run at 1 A for 1 hour. The

nitrocellulose membranes were blocked for 1 hour at room temperature in Tris-buffered

saline TTBS (400 ml 5X TBS: 2.5 M NaCl, 100 mM Tris, deionized H_2O; 1 ml Tween 20

and 1600 ml deionized H_2O) containing 5% fat-free milk followed by 3 rinses with TTBS

(15:10:5 min) prior to incubation overnight at 4°C in primary antibody. For GNMT a

1:40,000 dilution of polyclonal GNMT chicken IgY antibody (Aves Laboratory, #3809,

Tigard, Oregon) was used as the primary. For CBS a 1:400 dilution of rabbit IgG

polyclonal CBS antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the

primary. After rinsing with TTBS the nitrocellulose membranes were incubated at room

temperature for 1 h for CBS with goat-anti rabbit IgG horseradish peroxidase secondary

antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and GNMT with goat anti-

chicken IgY horseradish peroxidase secondary antibody (Aves Laboratory, Tigard,

Oregon). CBS and GNMT protein abundance was detected by chemiluminescence using

Kodak-Omat AR film. Densitometry analysis was performed using Quantity One

Software (Bio-Rad Laboratories).

Statistical Analysis

Statistical analysis was performed using Sigma Stat software (SPSS, Chicago,

IL). The mean values for each group were compared using two-way ANOVA at a

significance level of 5%. A one-way ANOVA on ranks was used if the normality test

failed. When the ANOVA was significant ($P < 0.05$), means were compared using

Fisher's least significant difference (LSD) procedure.

Results

Experiment 1

Creatine and genistein supplementation attenuated elevated plasma homocysteine concentrations induced by folate deficiency (Figure 2.2). Plasma homocysteine concentrations were increased by 95% over a 38-d period in rats receiving a folate deficient diet (Figure 2.1). Creatine supplementation (4 g/kg diet) for 10 d reduced plasma homocysteine concentrations (16%) in rats fed a folate-deficient diet ($p < 0.05$). Creatine supplemented, control rats tended to have reduced plasma homocysteine concentrations ($P = 0.08$). Genistein (300 mg/kg diet) supplementation did not reduce plasma homocysteine concentrations in control-fed rats, but a trend reduction ($P = 0.07$) existed in folate deficient rats fed genistein.

Creatine supplemented folate sufficient rats exhibited increased GNMT activity compared to folate deficient, creatine-supplemented rats (Figure 2.3). GNMT activity was significantly higher (23%; $P < 0.05$) in control, creatine supplemented rats compared with creatine-supplemented, folate-deficient rats. GNMT activity was not found to differ across the other treatment groups.

Experiment 2

Genistein supplementation attenuated elevated plasma homocysteine concentrations induced by folate deficiency (Figure 2.4). Plasma homocysteine concentrations were increased 106% in rats receiving a folate-deficient diet. Genistein (300 mg/kg diet) supplementation for 24 d reduced plasma homocysteine concentrations 24% in rats fed a folate deficient diet ($P < 0.05$). Genistein supplementation did not alter the plasma homocysteine concentrations of control fed rats.

Hepatic phosphatidylethanolamine N-methyltransferase (PEMT) activity was not altered by folate status or genistein supplementation (Figure 2.5). Rats were fed either a folate-sufficient or deficient diet containing genistein (300 mg/kg diet) for 24 d. Phosphatidylethanolamine N-methyltransferase (PEMT) activity was not found to differ across the treatment groups.

Hepatic methionine synthase (MS) activity was not altered by folate status or genistein supplementation (Figure 2.6). Rats were fed either a folate-sufficient or deficient diet containing genistein (300 mg/kg diet) for 24 d. Methionine synthase (MS) activity was not found to differ across the treatment groups.

Hepatic glycine N-methyltransferase (GNMT) activity was not altered by folate status or genistein supplementation (Figure 2.7). Rats were fed either a folate-sufficient or deficient diet containing genistein (300 mg/kg diet) for 24 d. Glycine N-methyltransferase (GNMT) activity was not found to differ across the treatment groups.

Hepatic glycine N-methyltransferase (GNMT) and cystathionine β-synthase (CBS) protein abundance were not altered by folate status or genistein supplementation (Figure 2.8). Rats were fed either a folate-sufficient or deficient diet containing genistein (300 mg/kg diet) for 24 d. Glycine N-methyltransferase (GNMT) and cystathionine β-synthase (CBS) protein abundance was not found to differ across the treatment groups.

Experiment 3

Genistein supplementation attenuated elevated plasma homocysteine concentrations induced by folate deficiency (Figure 2.9). Plasma homocysteine concentrations were increased by 243% over a 59-d period in rats receiving a folate-deficient diet. Genistein (300 mg/kg diet) supplementation for 59 d reduced plasma

homocysteine concentrations by 39% in rats fed a folate deficient-diet ($P < 0.05$) Genistein supplementation did not alter the plasma homocysteine concentrations of control-fed rats.

Hepatic phosphatidylethanolamine N-methyltransferase (PEMT) activity was not altered by folate status or genistein supplementation (Figure 2.10). Rats were fed either a folate-sufficient or deficient diet containing genistein (300 mg/kg diet) for 59 d. Phosphatidylethanolamine N-methyltransferase activity was not found to differ across the treatment groups.

Hepatic methionine synthase (MS) activity was not altered by folate status or genistein supplementation (Figure 2.11). Hepatic methionine synthase activity was not altered by folate status or genistein supplementation. Rats were fed either a folate-sufficient or deficient diet containing genistein (300 mg/kg diet) for 59 d. Methionine synthase activity was not found to differ across the treatment groups.

Hepatic glycine N-methyltransferase (GNMT) activity was not affected by folate status or genistein supplementation (Figure 2.12). Rats were fed either a folate-sufficient or deficient diet containing genistein (300 mg/kg diet) for 59 d. Hepatic GNMT activity was not found to differ across the treatment groups.

Renal glycine N-methyltransferase (GNMT) activity was not affected by folate status or genistein supplementation (Figure 2.13). Rats were fed either a folate-sufficient or deficient diet containing genistein (300 mg/kg diet) for 59 d. Renal GNMT activity was not found to differ across the treatment groups.

Genomic DNA methylation was not affected by folate status or genistein supplementation (Figure 2.14). Rats were fed either a folate-sufficient or deficient diet

containing genistein (300 mg/kg diet) for 59 d. Hepatic genomic methylation patterns were not found to differ across the treatment groups.

Hepatic glycine N-methyltransferase (GNMT) and cystathionine β-synthase (CBS) protein abundance were not altered by folate status or genistein supplementation (Figure 2.15). Rats were fed either a folate-sufficient or deficient diet containing genistein (300 mg/kg diet) for 59 d. Hepatic glycine N-methyltransferase and Cystathionine β-synthase protein abundance were not found to differ across the treatment groups.

Neither folate status or genistein supplementation altered gene expression of hepatic one-carbon metabolism enzymes (Table 2.2). Rats were fed either a folate-sufficient or deficient diet containing genistein (300 mg/kg diet) for 59 d. Expression of hepatic one-carbon metabolism regulatory enzymes was not found to differ across the treatment groups.

Neither folate status or genistein supplementation altered gene expression of hepatic DNA methyltransferase enzymes (Table 2.3). Rats were fed either a folate-sufficient or deficient diet containing genistein (300 mg/kg diet) for 59 d. Expression of hepatic DNA methyltransferase enzymes was not found to differ across the treatment groups.

Discussion

Genistein, a 17β-estradiol like phytoestrogen, exhibits a diverse array of biological activities, including modulatory actions influencing carcinogenesis and vascular pathogenesis (Marini et al., 2009). Soy isoflavones are consumed in varying quantities depending upon the population of interest. The molecular activities of isoflavones associated with disease prevention are complicated due to the diversity of human diets (Sacks et al., 2006). Additionally, the specificity of genistein toward estrogen receptors, namely the estrogen receptor beta (ER-β), elicits paradoxical effects as an agonist or antagonist, depending upon the tissue of interest and concentration of genistein treatment (Akiyama et al., 1987; Ye et al., 2009).

Previous research investigating the relationship between dietary genistein and one-carbon metabolism is limited. For the first time, we demonstrated through independent experiments that dietary administration of genistein at a high but achievable intake level (300 mg/kg diet) can attenuate plasma homocysteine concentrations during diet-induced folate deficiency.

S-adenosylmethionine (SAM), the universal methyl-donor, forms homocysteine upon methylating proteins, DNA, phospholipids, and other cellular constituents. Our initial work targeted creatine formation from guanidinoacetate via GAMT as a modulator of Homocysteine formation (Silva et al, 2008). Creatine supplementation reduces the endogenous demand on SAM-derived methyl groups, in turn reducing the production of Homocysteine (Silva et al., 2009). Our findings suggested that genistein could also independently lower plasma Homocysteine concentrations during diet-induced folate deficiency, although not as effectively as creatine supplementation. Unlike creatine

administration, which provides a product of SAM utilization, genistein does not directly provide a methyl-group for metabolism. Genistein may act independently through modification of the epigenome, including DNA methylation and histone acetylation, and these events may be intertwined with signaling via steroid receptors, namely estrogen receptors (Wietzke and Welsh, 2003; Cappelletti et al., 2006).

Our analysis of hepatic enzymes that regulate the production and degradation of Homocysteine imply that genistein is not altering the activity or expression of these enzyme targets. The subtle differences in GNMT activity demonstrated in experiment 2 (Figure 2.3) during genistein treatment were not supported by changes in protein abundance nor were they reproduced in experiment 3 (Figure 2.12, 2.13). Folate deficiency reorganizes the allosteric feedback mechanisms of one-carbon metabolites, including the inhibition of 5-methylTHF on GNMT and negative feedback of SAH on SAM and SAM on methionine adenosyltransferase (MAT) among others (Grillo and Colombatto, 2007). We would expect the regulation, activity, and production of these enzymes to change as folate depletion was exacerbated over time.

Chronic folate deficiency or diets lacking choline and methionine deplete cellular SAM causing sporadic cancers, including hepatocellular carcinoma, most plausibly due to perturbations of DNA methylation and misincorporation of uracil into DNA causing double strand breaks, chromosomal damage, and neoplasia (Pogribny et al., 2007). Substantially more time is required to produce pathogenesis in a model of moderate folate deficiency (Kim et al., 1994). Our findings that hepatic global methylation patterns were not altered by folate status at either 6 or 8 weeks and supports previous findings (Kim, 2004). Severe folate deficiency causes global hypomethylation through exhaustion

of SAM. However, rodent experiments have documented hypermethylated hepatic genomic DNA in animals fed a folate-deficient diet, suggesting hyperactivity of perturbed DNA methyltransferase enzymes (Kim, 2004). Genistein has been shown to maintain DNA methylation patterns in prostatic tissue, which expresses ER-β and at the same time have no effect on hepatic methylation status within the same animal (Day et al., 2002). The agouti mouse model experiments suggest that genistein is able to correct aberrations in fetal programming induced by a methyl-deficient diet, highlighting the potential activity of phytoestrogens during gestation (Dolinoy, et al., 2006). As an estrogen-like molecule, genistein may elicit stable alterations in gene expression at specific times across the lifespan.

Although the mechanisms are not completely understood, folate and genistein share similar activities related to chemoprevention, including tyrosine-kinase inhibition, which regulates cellular proliferation and apoptosis (Akiyama et al., 1987). In addition, both folate and genistein exhibit antioxidant properties, where folate scavenges oxidative radicals and genistein regulates oxidative damage at the level of the vascular endothelium in a similar fashion to estradiol (Billon-Gales et al., 2009). Therefore, although certainly unique, genistein and folate may overlap in certain regulatory aspects of vascular homeostasis and chemoprevention, including Homocysteine metabolism. Folate and genistein could mediate the same processes such as DNA methylation independently as shown in the agouti mouse model administered genistein during gestation with the ultimate effect of mediating one-carbon metabolism and attenuating elevated Homocysteine via an unknown mechanism (Dolinoy et al, 2006). Future research will aim to further classify the actions of genistein related to one-carbon metabolism.

Table 2.1. Forward and reverse primer sequences for analysis of candidate one-carbon

metabolism genes (Real-time PCR; Integrated DNA Technologies)

Genes	Forward Primers	Reverse Primers
GNMT	ACA ACA AAG CCC ACA TGG TAA CCC	AGC CGA AAC TTA CTG AAG CCA GGA
PEMT	TGT GCT CTC CAG CTT CTA TGC ACT	AGG GAA ATG TGG TCA CTC TGG ACT
MS	TTG GCC TAC CGG ATG AAC AAA TGC	AGC CAC AAA CCT CTT GAC TCC TGT
BHMT	ATC TGG GCA GAA GGT CAA TGA AGC	TGA CTC ACA CCT CCT GCA ACC AAT
CBS	AAC ATG TTG TCC TCC CTG CTT GCT	TCG GCT TGA ACT GCT TGT AGA GGA
Dnmt-1	TGT GGC AAG AAG AAA GGT GGC AAG	TGG ATG GAC TTG TGG GTG TTC TCA
Dnmt-3a	AGA GTG TCT GGA ACA CGG CAG AAT	TGC TGG TCT TTG CCC TGC TTT ATG
Dnmt-3b	TGC GCC TGC AAG ACT TCT TCA CTA	TGC AGG AAT CGC TGG GTA CAA CTT
18S (Ribosomal RNA)	CCA GAG CGA AAG CAT TTG CCA AGA	TCG GCA TCG TTT ATG GTC GGA ACT

Table 2.2. Relative gene expression of hepatic one-carbon metabolism enzymes in rats fed a folate sufficient (Ctrl) or deficient (FD) diet with or without genistein supplementation using the comparative C_T method. RNA was extracted from hepatic samples isolated from the same rats as described in Figure 2.9 and cDNA was synthesized as described under "Materials and Methods." Data is displayed as ΔC_t to indicate statistical analysis. The C_t value for each sample and gene of interest was normalized to the endogenous 18s housekeeping gene. Data are expressed as means ± S.E. (n = 6) and P-values of mean comparisons are labeled.

Enzyme Treatment	Mean Δ CT	P – Value	
GNMT			
Ctrl	14.3 ± 1.1	Diet	0.31
Ctr + G	11.3 ± 0.8	Genistein	0.32
FD	13.6 ± 1.3	Diet*Gen	0.12
FD + G	14.3 ± 1.4		
BHMT			
Ctrl	12.0 ± 0.6	Diet	0.23
Ctr + G	11.7 ± 0.2	Genistein	0.87
FD	12.3 ± 0.6	Diet*Gen	0.50
FD + G	12.8 ± 0.7		
CBS			
Ctrl	15.3 ± 0.5	Diet	0.41
Ctr + G	14.5 ± 0.5	Genistein	0.57
FD	14.5 ± 0.9	Diet*Gen	0.60
FD + G	15.4 ± 0.7		
MS			
Ctrl	17.7 ± 0.5	Diet	0.36
Ctr + G	17.7 ± 0.3	Genistein	0. 58
FD	17.9 ± 0.5	Diet*Gen	0.66
FD + G	18.3 ± 0.4		
PEMT			
Ctrl	13.2 ± 0.9	Diet	0.31
Ctr + G	11.2 ± 0.6	Genistein	0.42
FD	13.0 ± 1.2	Diet*Gen	0.25
FD + G	13.4 ± 1.1		

Table 2.3. Relative Gene Expression of hepatic DNA methyltransferase enzymes of rats

fed a folate sufficient or deficient diet with or without genistein supplementation using

the comparative C_t method. RNA was extracted from hepatic samples isolated from the

same rats as described in Figure 2.9 and cDNA was synthesized as described under

"Materials and Methods." Data is displayed as ΔC_t to indicate statistical analysis. The C_t

value for each sample and gene of interest was normalized to the endogenous 18s

housekeeping gene. Data are expressed as means ± S.E. (n = 6) and P-values of mean

comparisons are labeled.

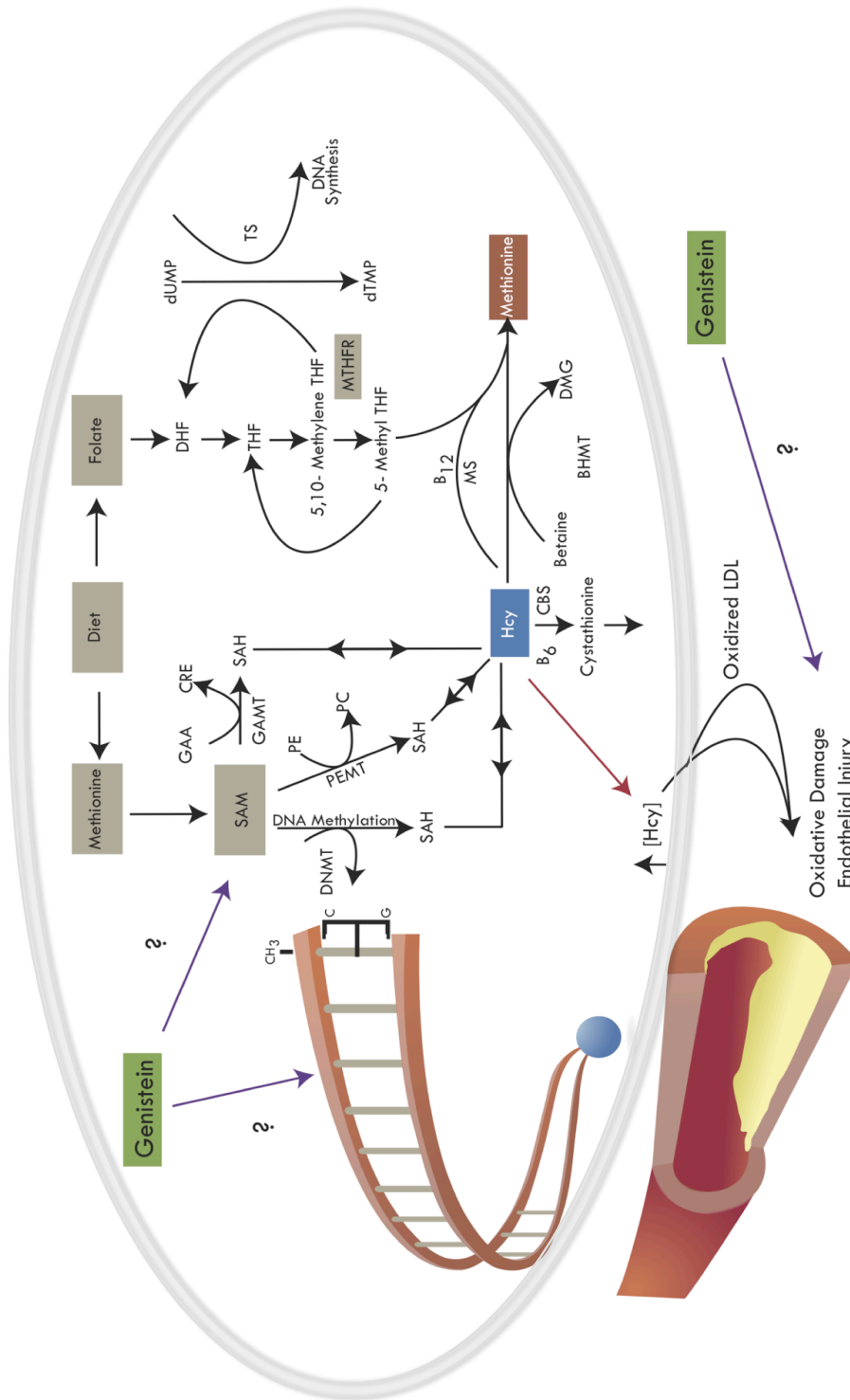| Enzyme Treatment	Mean Δ CT	P – Value	
Dnmt-1			
Ctrl	19.4 ± 0.6	*Diet*	0.4
Ctr + G	19.0 ± 0.2	*Genistein*	0.9
FD	19.5 ± 0.6	*Diet*Gen*	0.4
FD + G	19.9 ± 0.6		
Dnmt-3a			
Ctrl	18.5 ± 0.7	*Diet*	0.4
Ctr + G	17.3 ± 0.3	*Genistein*	0.5
FD	18.3 ± 0.6	*Diet*Gen*	0.3
FD + G	18.6 ± 0.8		
Dnmt-3b			
Ctrl	19.1 ± 0.5	*Diet*	0.2
Ctr + G	18.9 ± 0.4	*Genistein*	0.7
FD	19.8 ± 0.5	*Diet*Gen*	0.9
FD + G	19.5 ± 0.4		

Figure 2.1. One carbon metabolism and the isoflavone genistein.

Figure 2.1. One-carbon metabolism and the isoflavone genistein. S-adenosylmethionine (SAM) is the major biological methyl group donor. SAM-derived methyl groups are allocated towards the formation of metabolites, including creatine (Cre) and phosphatidylcholine (PC), and for DNA methylation. Tetrahydrofolate (THF) coenzymes are required for nucleotide biosynthesis and for the endogenous remethylation of homocysteine to form methionine. Homocysteine accumulates in the absence of 5-methyltetrahydrofolate and must be either remethylated back to methionine in the absence of folate via betaine-homocysteine methyltransferase (BHMT), or irreversibly catabolized via the transsulfuration pathway (e.g., cystathionine β-synthase; CBS). Hyperhomocysteinemia is an independent risk factor for vascular disease and may reflect perturbed cellular methylation potential, which influences DNA methylation, an epigenetic phenomenon. Genistein may modulate the formation and degradation of Homocysteine, by regulating SAM metabolism, DNA methylation processes, and through its paradoxical actions as an estrogen receptor agonist and antagonist. Additional abbreviations used are: DHF, dihydrofolate; dUMP, deoxyuridine; dTMP, deoxythymidine; TS, thymidylate synthase; GAA, guanidinoacetate; GAMT, guanidinoacetate methyltransferase; DMG. Dimethylglycine; SAH, S-adensylhomocysteine; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; Dnmt, DNA methyltransferase; PE, phosphatidylethanolamine. Question marks (?) indicate undetermined but proposed interactions of genistein.
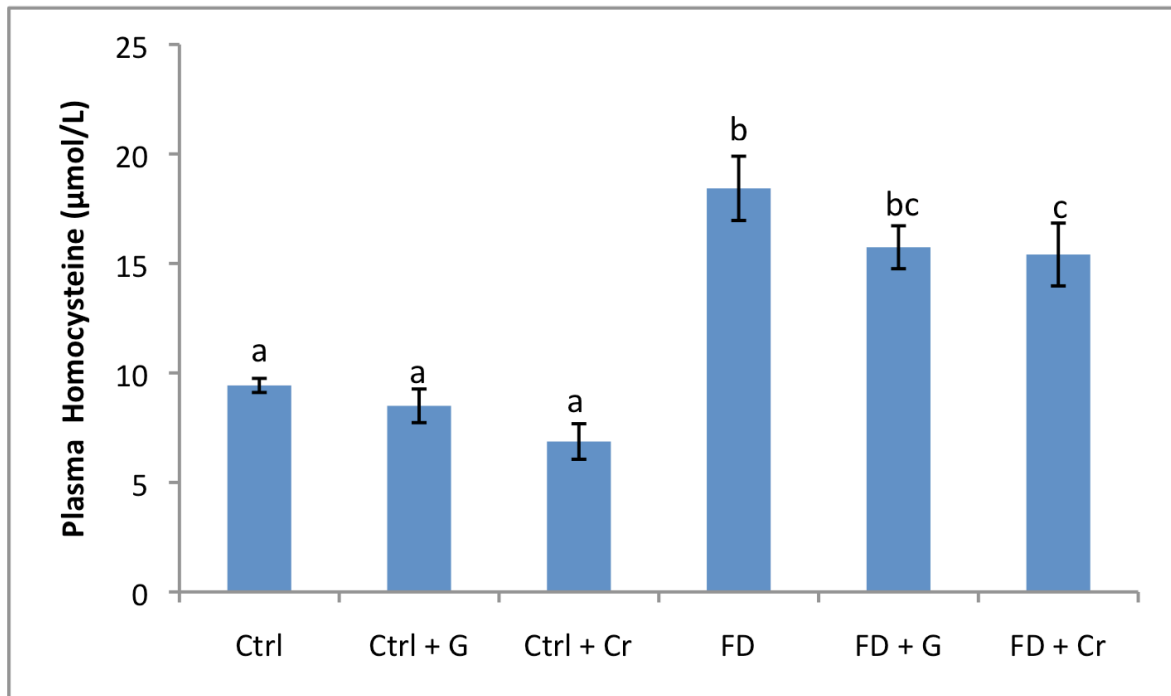
Figure 2.2. Plasma homocysteine concentrations from rats fed a folate sufficient (Ctrl) or deficient diet (FD) with or without genistein (G) and creatine (Cr) supplementation. Male Sprague Dawley rats were obtained at 3-4 wk of age (50-74g) and fed for 28 d on a diet containing, 10% vitamin free casein, 0.3% L-methionine, 40.2% corn starch, 39.3 % dextrose, 5% corn oil, 4% mineral mix, 2% choline bitartrate (1.5 M), and 1% vitamin mix with either 2 ppm folate (folate-sufficient, Ctrl), or 0 ppm folate (folate-deficient, FD) for 28 d. On 29 d, parallel diets supplemented with genistein (300mg/kg diet; Ctrl + G and FD + G) or 4g/kg diet creatine monohydrate (Ctrl + Cre and FD + Cre) were fed for an additional 10 d. On 38 d, blood samples were collected via cardiac puncture, centrifuged, and plasma fractions were prepared and analyzed using HPLC, as described under "Materials and Methods." Data are expressed as means ± S.E. (n = 6). Bars with different letters denote means that differ ($P < 0.05$).
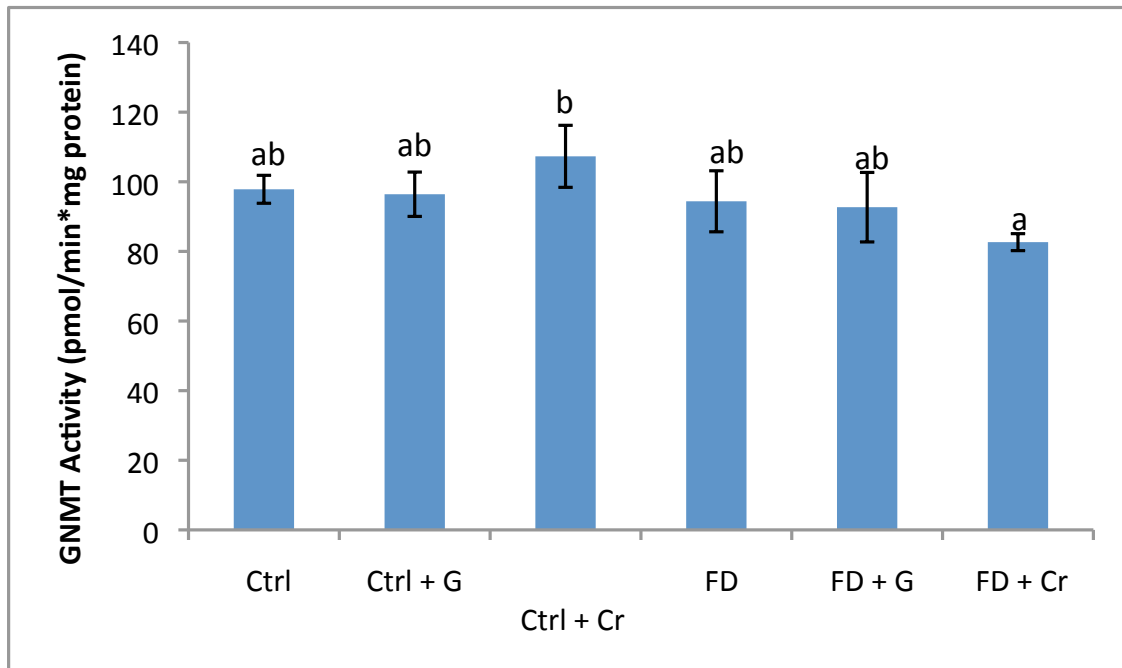
Figure 2.3. Glycine N-methyltransferase (GNMT) activity of rats fed a folate sufficient (Ctrl) or deficient (FD) diet with or without genistein and creatine supplementation. Hepatic samples from the same rats as described in Figure 2.2 were isolated and used to determine glycine N-methyltransferase activity as described under "Materials and Methods." Data are expressed as means ± S.E. (n = 6). Bars with different letters denote means that differ (P < 0.05).
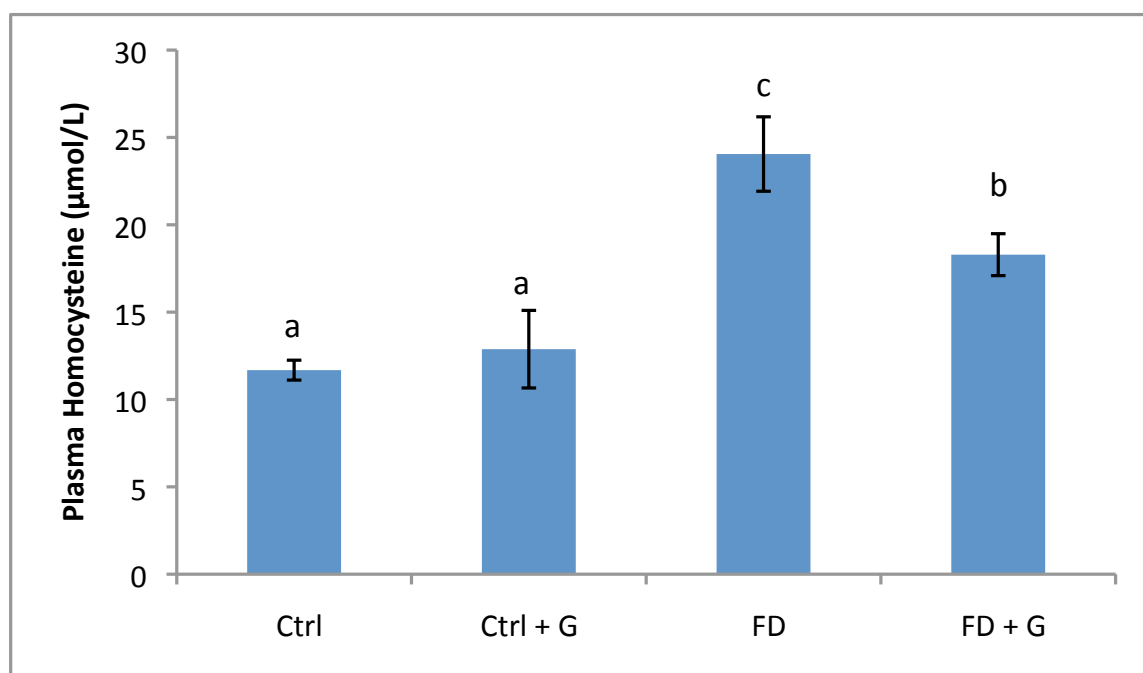
Figure 2.4. Plasma homocysteine concentrations of rats fed a folate sufficient (Ctrl) or deficient diet (FD) with or without genistein supplementation. Male Sprague Dawley rats were obtained at 3-4 wk of age (50-74g) and fed for 28 d on a diet containing, 10% vitamin free casein, 0.3% L-methionine, 40.2% corn starch, 39.3 % dextrose, 5% corn oil, 4% mineral mix, 2% choline bitartrate (1.5 M), and 1% vitamin mix with either 2 ppm folate (folate-sufficient, Ctrl), or 0 ppm folate (folate-deficient, FD) for 14 d prior to supplementation with genistin (300 mg/kg diet) for an additional 24 d. On 38 d, blood samples were collected via cardiac puncture, centrifuged, and plasma fractions were prepared and analyzed using HPLC, as described under "Materials and Methods." Data are expressed as means ± S.E. (n = 6). Bars with different letters denote means that differ (P < 0.05).
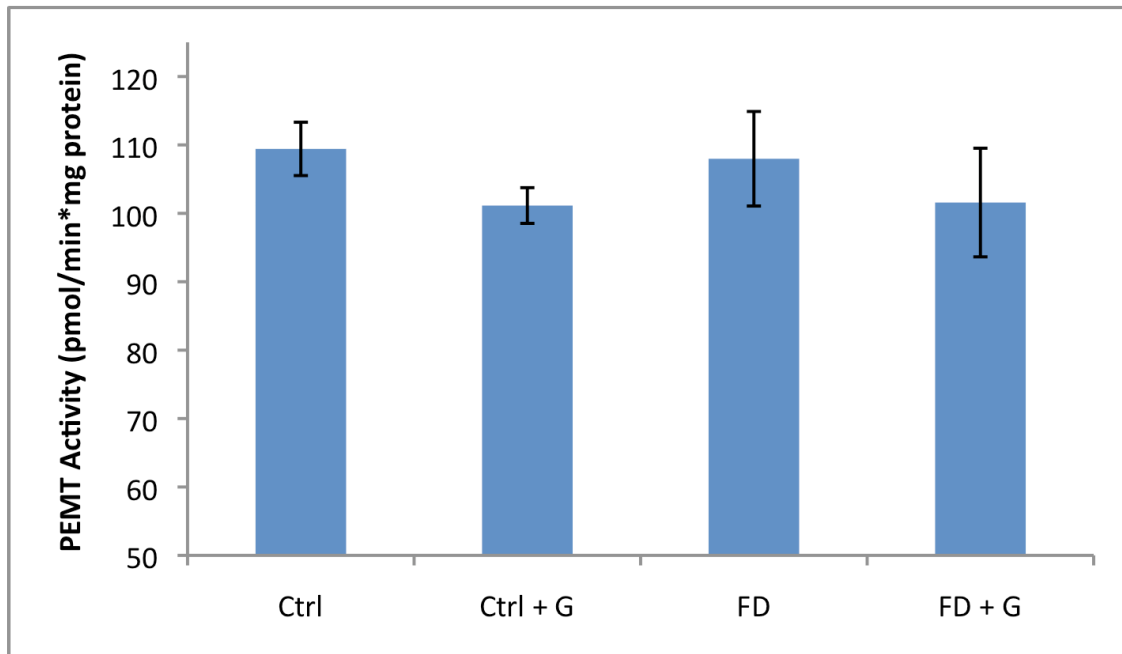
Figure 2.5. Phosphatidylethanolamine N-methyltransferase (PEMT) activity of rats fed a folate-sufficient (Ctrl) or deficient diet (FD) with or without genistein supplementation. Hepatic samples from the same rats as described in Figure 2.4 were isolated and used to determine phosphatidylethanolamine N-methyltransferase (PEMT) activity as described under "Materials and Methods." Data are expressed as means ± S.E. (n = 6).
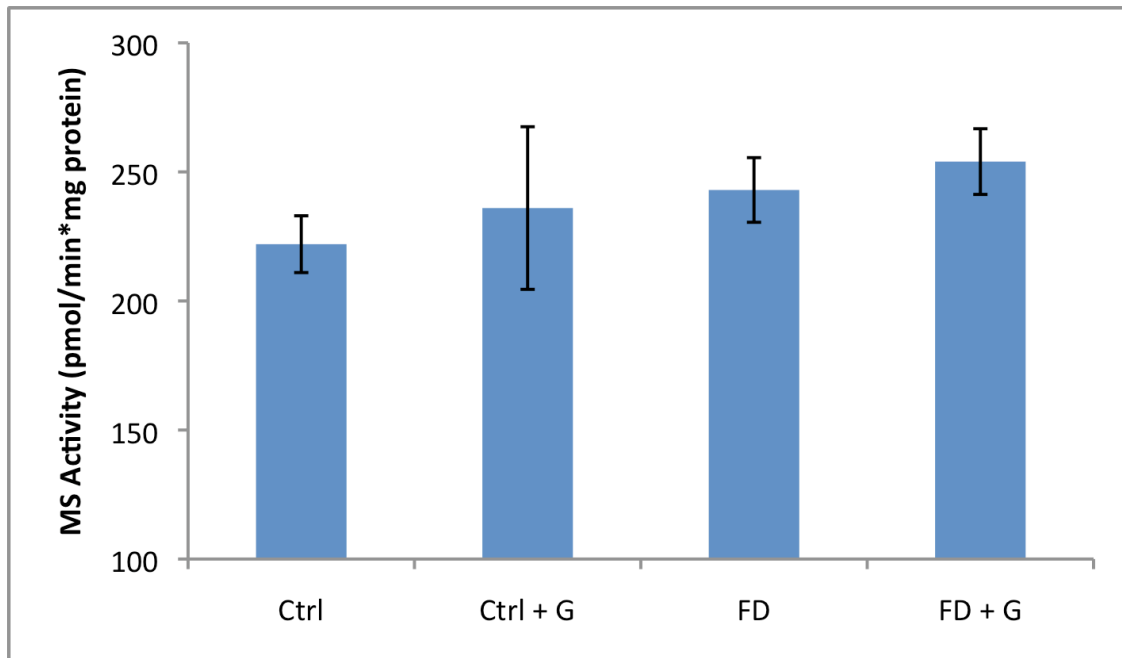
Figure 2.6. Methionine synthase (MS) activity of rats fed a folate sufficient (Ctrl) or deficient diet (FD) with or without genistein supplementation. Hepatic samples from the same rats as described in Figure 2.4 were isolated and used to determine methionine synthase (MS) activity as described under "Materials and Methods." Data are expressed as means ± S.E. (n = 6).
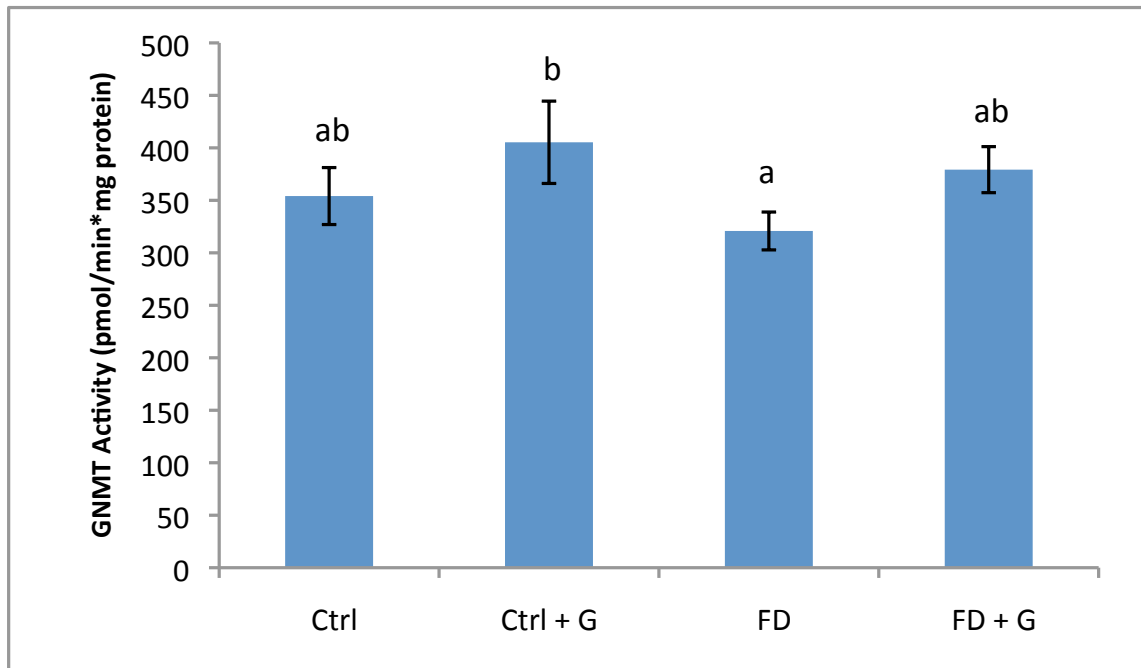
Figure 2.7. Glycine N-methyltransferase (GNMT) activity of rats fed a folate sufficient (Ctrl) or deficient (FD) diet with or without genistein supplementation. Hepatic samples from the same rats as described in Figure 2.4 were isolated and used to determine glycine N-methyltransferase activity as described under "Materials and Methods." Data are expressed as means ± S.E. (n = 6). Bars with different letters denote means that differ (P < 0.05).
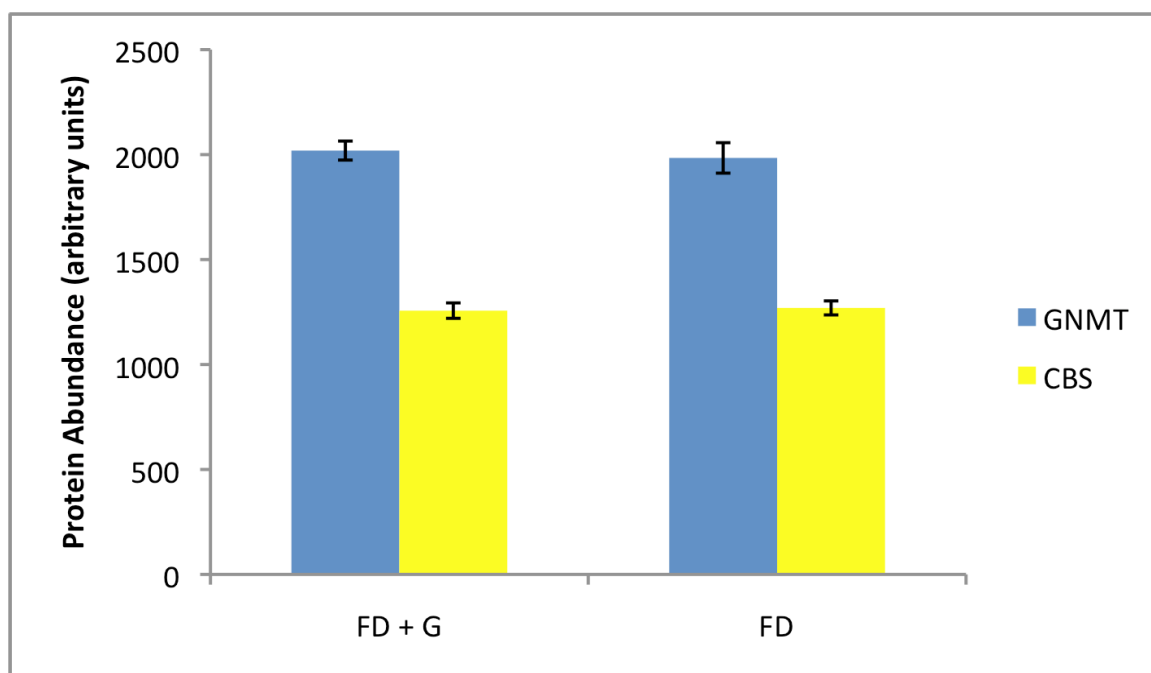
Figure 2.8. Glycine N-methyltransferase (GNMT) and Cystathionine β-synthase (CBS) protein abundance of rats fed a folate-sufficient (Ctrl) or deficient (FD) diet with or without genistein supplementation. Hepatic samples from the same rats as described in Figure 2.4 were isolated and used to determine glycine N-methyltransferase (GNMT) and cystathionine β-synthase protein abundance by Western blot analysis as described under "Materials and Methods." Data are expressed as means ± S.E. (n = 6).
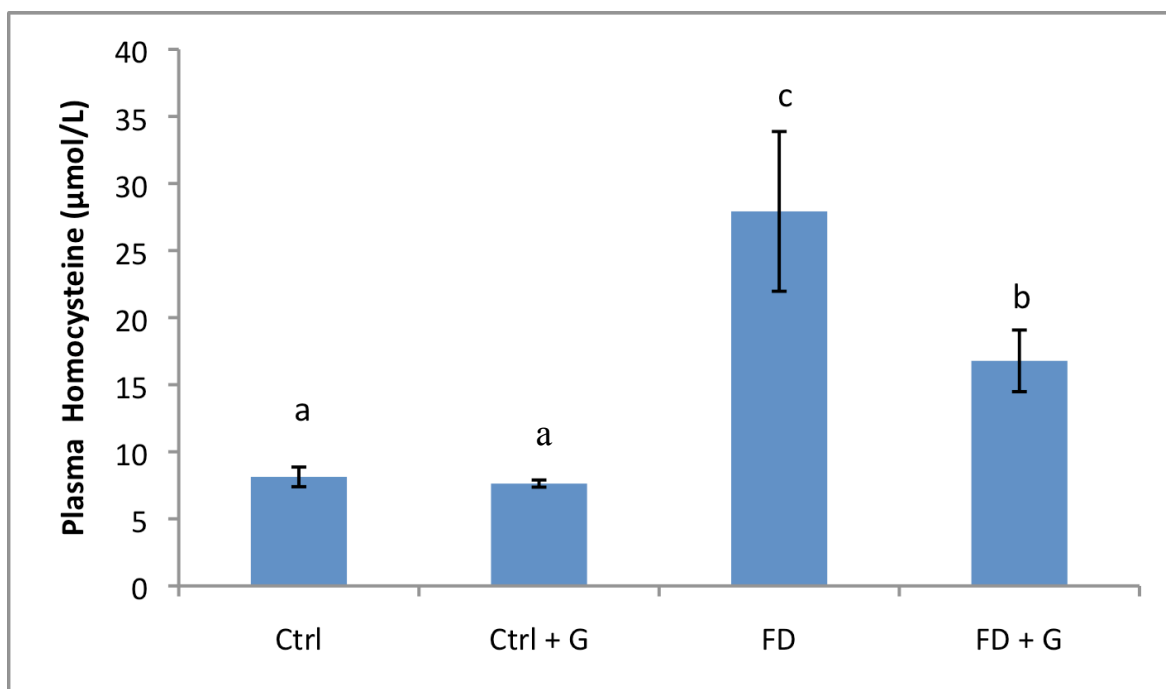
Figure 2.9. Plasma homocysteine concentrations of rats fed a folate sufficient (Ctrl) or deficient diet (FD) with or without genistein supplementation. Male Sprague Dawley rats were obtained at 3-4 wk of age (50-74g) and fed for 5 d on a diet containing, 10% vitamin free casein, 0.3% L-methionine, 40.2% corn starch, 39.3 % dextrose, 5% corn oil, 4% mineral mix, 2% choline bitartrate (1.5 M), and 1% vitamin mix with 2 ppm folate (folate sufficient, Ctrl). On 6 d rats were either maintained on the Ctrl diet or folate-deficient diet (FD, 0 ppm folate) with or without genistein (300 mg/kg diet) for an additional 59 d. On 64 d, blood samples were collected via cardiac puncture, centrifuged, and plasma fractions were prepared and analyzed using HPLC, as described under "Materials and Methods." Data are expressed as means ± S.E. (n = 6). Bars with different letters denote means that differ ($P < 0.05$).

Figure 2.10. Phosphatidylethanolamine N-methyltransferase (PEMT) activity of rats fed a folate-sufficient (Ctrl) or deficient diet (FD) with or without genistein supplementation. Hepatic samples from the same rats as described in Figure 2.9 were isolated and used to determine phosphatidylethanolamine N-methyltransferase (PEMT) activity as described under "Materials and Methods." Data are expressed as means ± S.E. (n = 6).

Figure 2.11. Methionine synthase (MS) activity of rats fed a folate sufficient (Ctrl) or deficient (FD) diet with or without genistein supplementation. Hepatic samples from the same rats as described in Figure 2.9 were isolated and used to determine methionine synthase (MS) activity as described under "Materials and Methods." No significant differences were found in MS activity between folate status or genistein supplementation. Data are expressed as means ± S.E. (n = 6).
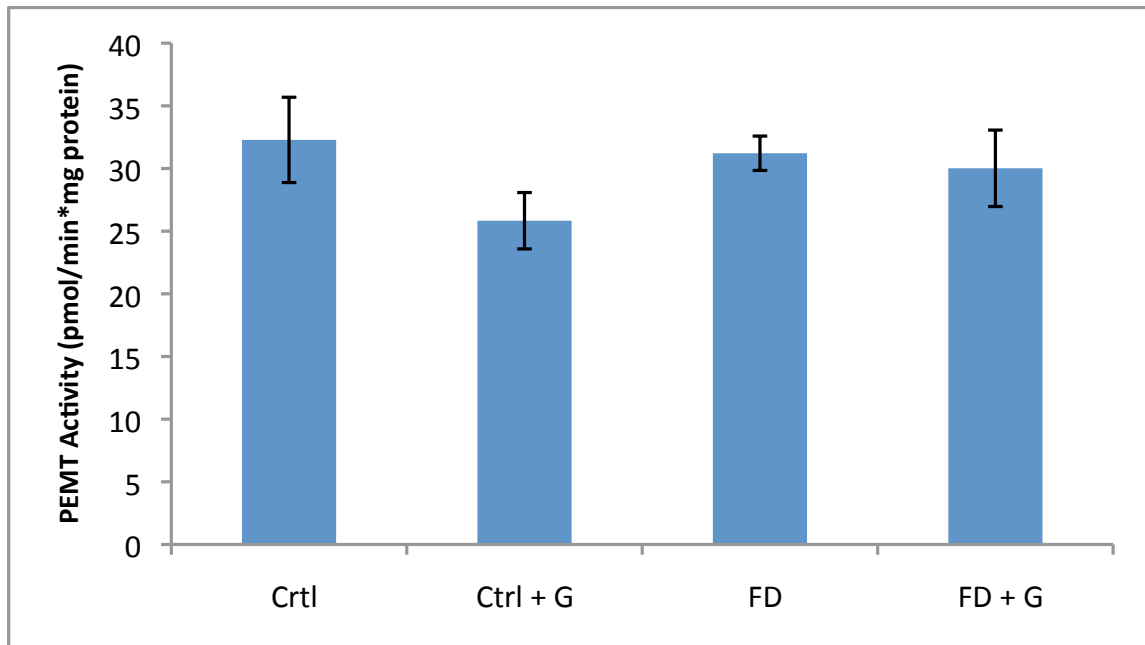
Figure 2.12. Hepatic glycine N-methyltransferase (GNMT) activity of rats fed a folate-sufficient (Ctrl) or deficient (FD) diet with or without genistein supplementation. Hepatic samples from the same rats as described in Figure 2.9 were isolated and used to determine hepatic glycine N-methyltransferase activity as described under "Materials and Methods." Data are expressed as means ± S.E. (n = 6).

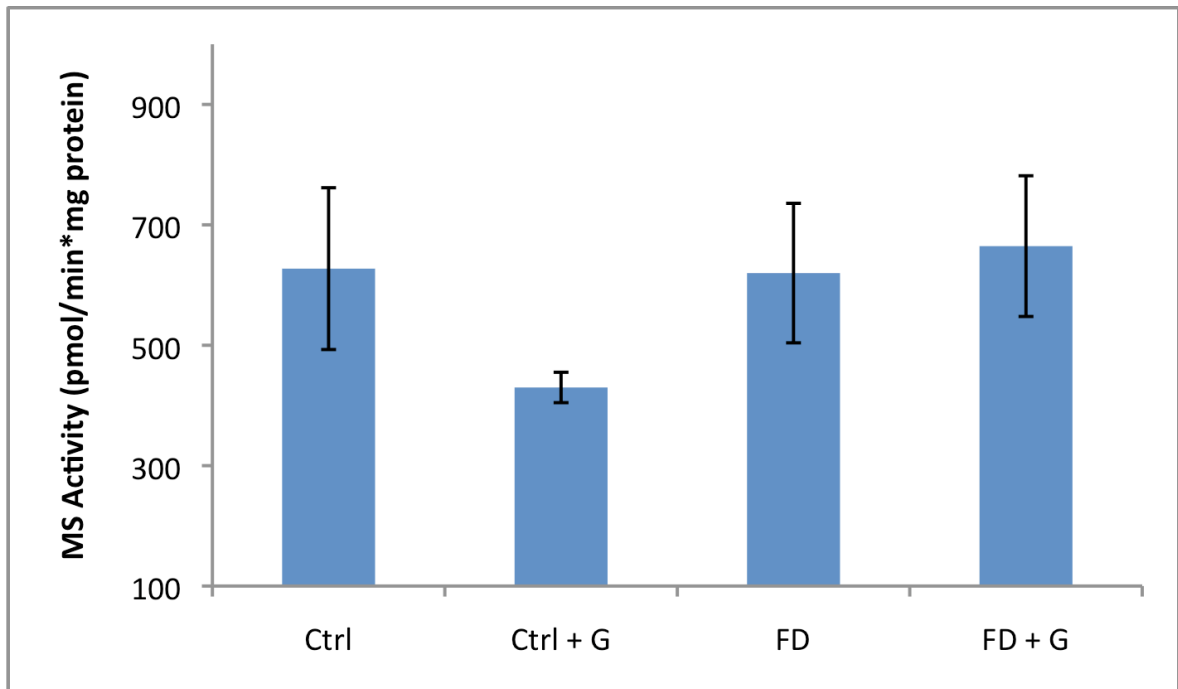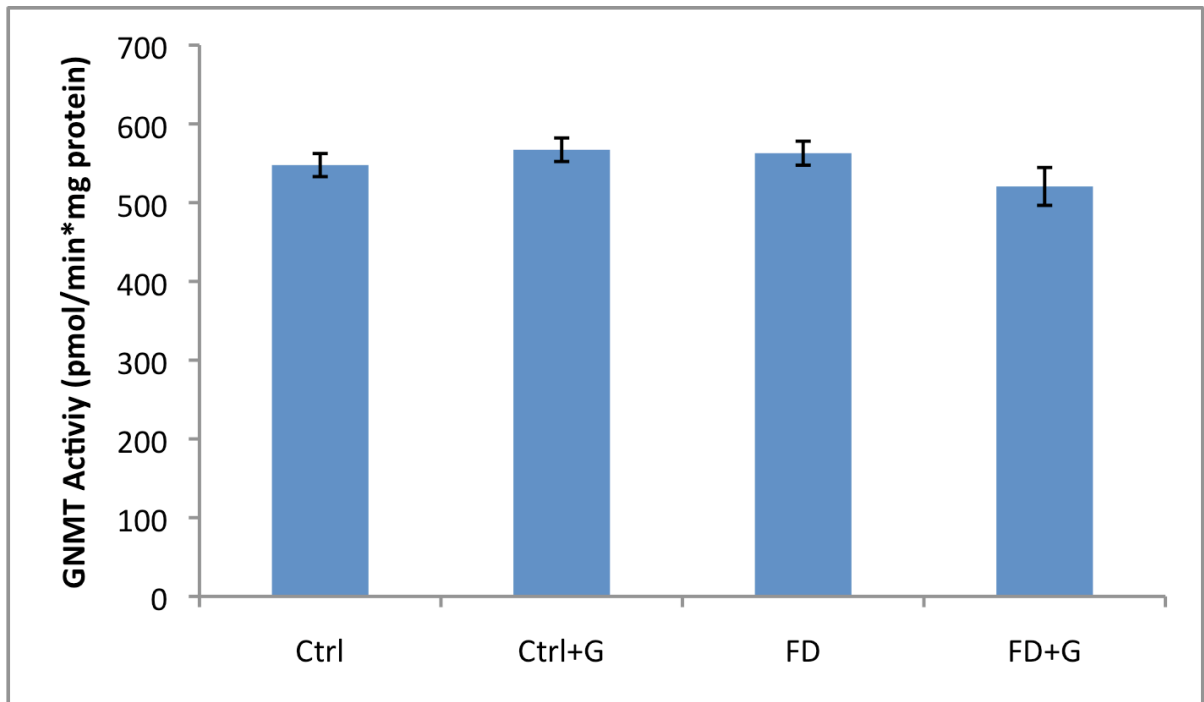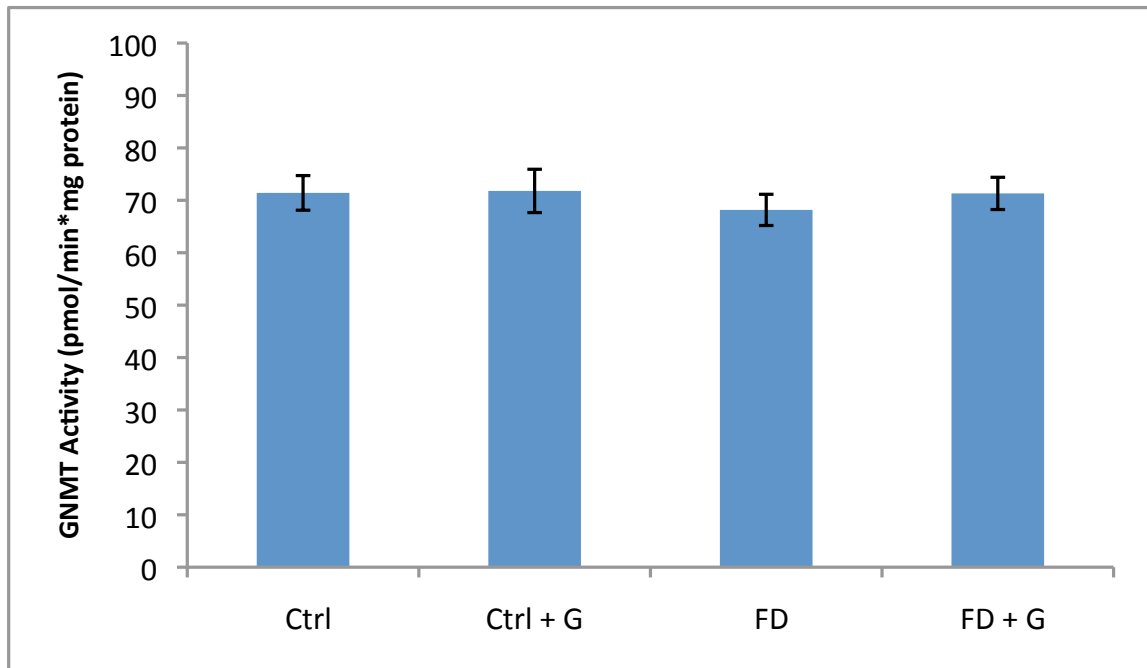Figure 2.13. Renal glycine N-methyltransferase (GNMT) activity of rats fed a folate-sufficient (Ctrl) or deficient (FD) diet with or without genistein supplementation. Whole kidney samples from the same rats as described in Figure 2.9 were excised and prepared to determine renal glycine N-methyltransferase (GNMT) activity as described under "Materials and Methods." Data are expressed as means ± S.E. (n = 6).

Figure 2.14. Genomic DNA methylation of rats fed a folate-sufficient (Ctrl) or deficient (FD) diet with or without genistein supplementation. Hepatic samples from the same rats as described in Figure 2.9 were obtained and DNA was isolated to determine genomic DNA methylation as described under "Materials and Methods." Data are expressed as means ± S.E. (n = 6).

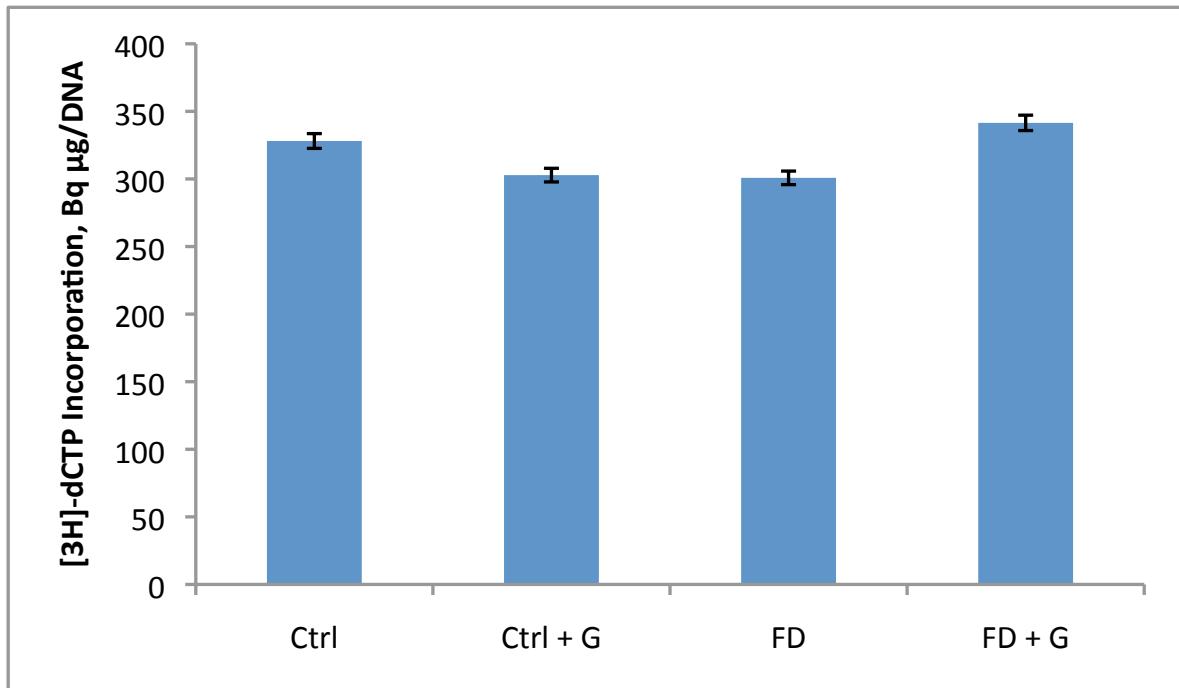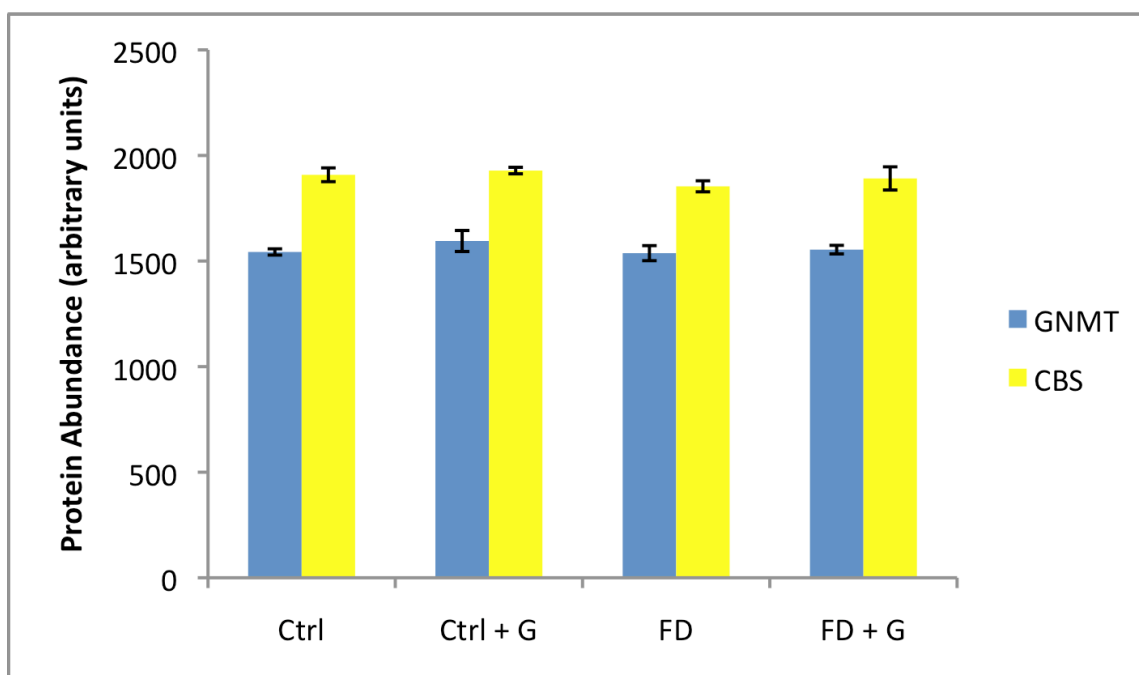Figure 2.15. Glycine N-methyltransferase (GNMT) and cystathionine β-synthase (CBS) protein abundance of rats fed a folate-sufficient (Ctrl) or deficient (FD) diet with or without genistein supplementation. Hepatic samples from the same rats as described in Figure 2.9 were isolated and used to determine glycine N-methyltransferase (GNMT) and cystathionine β-synthase protein abundance by Western blot analysis as described under "Materials and Methods." Data are expressed as means ± S.E. (n = 6).

CHAPTER 3. GENERAL CONCLUSIONS

In this thesis, I have elucidated further the actions of the bioactive phytoestrogen genistein in relation to perturbations in one-carbon metabolism caused by diet induced folate deficiency. Chronic folate deficiency poses significant risks that include vascular disease, neurological disorders, birth defects, certain cancers, and diminished physiological performance due to anemia and growth retardation (Beaudin and Stover, 2007). Mandatory fortification of cereals and grains in the late 1990s dramatically reduced the incidence of neural tube defects and also increased the circulating concentration of 5-methylTHF across the population of the United States. However, folate status varies among individuals and particularly within those who display polymorphic variation in the genes regulating folate metabolism such as those encoding methylene tetrahydrofolate reductase (MTHFR) and cystathionine β-synthase (CBS). Gene variation in the MTHFR enzyme is believed to exist in upwards of 15% of the population and the associated risk factors appear to be highly dependent upon folate status as demonstrated through reduced enzyme function (Dowey et al., 2004).

Homeostatic one-carbon metabolism is dependent upon a number of substrates and cofactors other than folate that include vitamins B_{12}, B_6, and B_2 and the methyl-providers betaine, choline, and methionine (Verhoef et al., 1996). There are numerous ways to perturb cellular methylation potential in an experimental model, including providing a diet with a defined amino acid profile or depleting the diet of any of the multiple aforementioned constituents (Wilcken and Wilcken, 1998). Our research model produced a state of moderate folate deficiency through depletion of dietary folate without antibiotic treatment. Antibiotics prevent folate synthesis by gut flora exacerbating folate

losses and depleting hepatic choline pools due in part to increased folate independent remethylation of homocysteine via betaine homocysteine methyltransferase (BHMT). Hepatic choline and phosphatidylcholine stores are reduced by 65% and 85% respectively at 4 weeks in animals fed a folate deplete diet with antibiotics. However, rats subjected to moderate folate deficiency exhibit only a 30% reduction in hepatic choline stores at 24 weeks (Kim et al, 1994). Severe folate deficiency is now rare in industrialized countries, and our model aimed to represent a subset of the population with altered folate metabolism or inconsistent intake.

In each of my studies we demonstrated a consistent and dramatic increase in plasma homocysteine concentration caused by folate restriction without changes in enzyme function. Plasma homocysteine concentrations reflect the global folate metabolism of an animal, including transmethylation, remethylation, and transsulfuration pathways (Hazra et al., 2009). The liver accounts for nearly 80% of SAM utilization, and although I did not see changes in expression or abundance of one-carbon metabolism enzymes, we expect that ultimately this tightly regulated network would reflect depleted folate pools. For example, 5-methylTHF is an allosteric inhibitor of glycine N-methyltransferase (GNMT) and, therefore folate deficiency, would eventually alleviate this inhibition. Similarly, CBS activity would increase to enhance the catabolism of accumulating homocysteine within the cell. Folate independent remethylation of homocysteine via BHMT would compensate for the reduced formation of 5-methylTHF, depleting choline pools and altering the activity of phosphatidylethanolamine N-methyltransferase (PEMT), a major consumer of SAM-derived methyl groups (Wagner, 2005).

Hyperhomocysteinemia is an independent risk factor for cardiovascular disease (Aguilar et al., 2004). Homocysteine promotes endothelial damage, platelet aggregation, thrombosis, inflammation, and lipid/LDL oxidation. The associated rise of homocysteine, common in vascular disease patients, is not completely understood and may actually represent a marker for more severe cellular alterations including reduced cellular methylation potential, which causes DNA hypomethylation, double strand breaks, and chromosomal damage (Duthie et al., 2002). In my preliminary study, I hypothesized that supplementation with creatine monohydrate would reduce the endogenous demand on SAM in a folate deficient model. The work done by Brosnan and colleagues demonstrated that administration of guanidinoacetate increases plasma homocysteine concentrations, while creatine supplementation attenuates basal homocysteine concentrations (Stead et al., 2001). To our knowledge, we are the first to demonstrate that creatine supplementation can decrease plasma homocysteine concentrations in a folate-deficient rat model. We speculate that this effect is mediated through reduced activity of hepatic guanidinoacetate methyltransferase (GAMT) and renal L-arginine: glycine amidinotransferase, which form creatine and guanidinoacetate respectively (Silva et al., 2008).

During our initial work we also investigated the effects of genistein administration on one-carbon metabolism. Previous research indicates that genistein can alter methylation patterns in the agouti mouse model fed a methyl-deficient diet, preventing fetal imprinting (e.g., hypomethylation) which predisposes the animal to various disease states (Dolinoy et al., 2006). Soy isoflavones have been implicated in the prevention of various diseases, largely based on differences between population studies

or alternately ex vivo work with pharmacological treatments. The role of phytoestrogens in disease prevention is controversial due to both the multitude of confounding factors of a normal human diet (i.e., soy protein and lipid composition) and the potential deleterious actions of estrogen-like compounds, including the promotion of estrogen-responsive cancers (Ye et al., 2009). We have demonstrated that genistein is able to reduce hyperhomocysteinemia caused by diet-induced folate deficiency across various time points of folate restriction and genistein supplementation. We have yet to classify a consistent modification of the enzymes regulating one-carbon metabolism, which would cause the reduction in plasma homocysteine concentrations. Conceivably, genistein may be acting at a greater efficiency in estrogen-responsive tissues such as the testes and prostate, where ER-β is expressed at a higher level than within the liver (Cappelletti et al, 2006). It is also possible that genistein may be functioning to a greater extent in the kidney, where a significant portion of SAM metabolism occurs. My major work involved the investigation of hepatic transmethylation, remethylation, and transsulfuration enzymes, however, many questions need to be addressed in future research.

First and foremost, I have not yet determined the metabolic target of genistein as it relates to homocysteine metabolism. Research has demonstrated that genistein may alter the activity of DNA methyltransferase enzymes, which although certainly relevant, does not completely explain the considerable reduction in plasma homocysteine, as total DNA methylation is believed to account for less than 20% of SAM utilization. My analysis of hepatic global methylation did not shed light on this interaction because our model of folate deficiency did not induce hepatic genomic hypomethylation. However, it

is possible that genistein is acting at a more specific level than genomic methylation maintenance or at a higher affinity in estrogen-responsive tissues. Genistein could be indirectly regulating one-carbon metabolism as an estrogen-like molecule, independently of folate. The structural nature of genistein allows it to interact not only with ER-β but also the vitamin D receptor (VDR) and the retinoid X receptor (RXR) (Weitzke and Welsh, 2003). Future research should investigate potential hormonal interactions to determine if genistein is acting via alternative mechanisms.

It would be interesting to conduct a similar experiment using a fetal-programming model, where pregnant rats are fed a folate-restricted diet supplemented with genistein and transmethylation, remethylation, and transsulfuration enzyme function was evaluated as well as alterations in DNA methylation in and beyond hepatic tissue. Based on the agouti mouse studies demonstrating alterations in the epigenome independent of folate, I could determine if these changes alter methionine metabolism or specifically DNA methyltransferases (Dolinoy et al., 2006). It appears that in utero or early post-natal exposure to environmental factors and nutrients plays a pivotal role in the development and disease susceptibility of an animal and these effects become diluted as the animal ages (Liu et al., 2008). Targeting specific genes of interest would provide a greater understanding of early epigenetic regulation by genistein. It is possible that the regulatory elements of enzymes functioning to mediate one-carbon metabolism are within those influenced by genistein or distant regulation is modulating the enzymes of interest, as DNA methylation and histone acetylation regulate transcription beyond their immediate locations (Stover, 2009). I have demonstrated for the first time that genistein is able to attenuate plasma homocysteine concentrations in rats caused by dietary folate

depletion. My future work will aim to determine how genistein modifies the production

and or degradation homocysteine through its mediation of one-carbon metabolism.

ACKNOWLEDGEMENTS

I am sincerely grateful to those who encouraged, guided, and helped during my time at Iowa State University. I would like to extend my gratitude and appreciation to my major professor Dr. Kevin Schalinske who provided me with the opportunity to pursue graduate education in his laboratory. I am very grateful for the opportunities he has provided me, and for the knowledge I have gained under his guidance as a young scientist and a person. Dr. Schalinske has promoted my development both in and beyond the laboratory and I will undoubtedly benefit from these experiences for the remainder of my career.

I would like to thank the members of my program of study committee, Dr. Donald Beitz and Dr. Michael Spurlock, for their input, time, and effort helping me develop during my graduate studies. I am very grateful for their critique of my work and examples on how to improve as a student and scientist. I am also very appreciative of the opportunity to rotate and learn in the laboratory of Dr. Spurlock and for his guidance and mentoring.

I am also very appreciative of those who helped me throughout my graduate studies. As an incoming and developing student in Dr. Schalinske's laboratory, Dr. Kristin Nieman, Dr. Kelly T. Williams, Stacy Schroeder, Cara H. Nelson, Ginny Knoblock, and Joshua Neuman were essential components of my research on a daily basis. These individuals demonstrated nothing but the utmost support of my work and development in the laboratory, and treated me with great kindness. I am very grateful for the hours of time they spent helping me with my projects.

I would like to especially thank Dr. Matthew Rowling, Dr. Nicholas Gabler, Dr. Jennifer Walker-Daniels, Dr. Jeremy Davis, Doug Braucher, Rebecca L. Boddicker, Richard Faris, Nan Huang, and Rachel Anderson for providing additional input and help regarding my research methods and techniques. I have benefited tremendously from the collaborative environment of our faculty and student group.

I am very fortunate to have had the opportunity to be a graduate student in the Interdepartmental Graduate Program in Nutritional Sciences and the Department of Food Science and Human Nutrition. I have had excellent opportunities and benefited tremendously from the advice, guidance, and character modeling of the faculty and my peers.

I am very grateful for the support from my family and friends. My parents, Brian and Gail, have always supported my interests and provided me with endless opportunities. I owe my passion of science to my parents and will be forever grateful for their patience, support, and advice during my time at Iowa State University. I hope that I can model their hard work ethic, logic, and kindness.

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