Exercise prevents hyperhomocysteinemia in a folate-deficient mouse model

Joshua Charles Neuman

Iowa State University

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Exercise prevents hyperhomocysteinemia in a folate-deficient mouse model

by

Joshua Charles Neuman

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:
Kevin Schalinske, Major Professor
Matthew Rowling
Marian Kohut

Iowa State University
Ames, Iowa
2010

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DEDICATION

I would like to dedicate this thesis to both of my grandfathers John H. Hammersmith and Paul Neuman. Although they are no longer with us, their wisdom and guidance were essential components to my success in seeking and completing my master’s degree and thesis. I am grateful for their teachings early in my life and will take them with me as I pursue my future goals.
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<th>Full Form</th>
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<tr>
<td>5-CH$_3$-THF</td>
<td>5-methyltetrahydrofolate (monoglutamate form)</td>
</tr>
<tr>
<td>5-CH$_3$-THFn</td>
<td>5-methyltetrahydrofolate (polyglutamate form)</td>
</tr>
<tr>
<td>5,10-CH$_2$-THF</td>
<td>5,10-methylene tetrahydrofolate</td>
</tr>
<tr>
<td>AGAT</td>
<td>L-arginine-glycine amidinotransferase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BHMT</td>
<td>Betaine-homocysteine S-methyltransferase</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CBS</td>
<td>Cystathionine beta-synthase</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CGL</td>
<td>Cystathionine $\gamma$-lyase</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphate-guanine</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DHF</td>
<td>Dihydrofolate</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>DMG</td>
<td>Dimethylglycine</td>
</tr>
<tr>
<td>DNMTs</td>
<td>DNA methyltransferases</td>
</tr>
<tr>
<td>dTMP</td>
<td>Deoxythymidine-5-monophosphate</td>
</tr>
<tr>
<td>dUMP</td>
<td>Deoxyuridine-5-monophosphate</td>
</tr>
<tr>
<td>GAMT</td>
<td>Guanidinoacetate $N$-methyltransferase</td>
</tr>
<tr>
<td>GNMT</td>
<td>Glycine $N$-methyltransferase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>ICR</td>
<td>Imprinting control region</td>
</tr>
<tr>
<td>LSD</td>
<td>Least significant difference</td>
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<tr>
<td>MAT</td>
<td>Methionine adenosyltransferase</td>
</tr>
<tr>
<td>MS</td>
<td>Methionine synthase</td>
</tr>
<tr>
<td>MT(s)</td>
<td>Methyltransferases</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Metylenetetrahydrofolate reductase</td>
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<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>NTD(s)</td>
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<tr>
<td>p53</td>
<td>Tumor protein 53</td>
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<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEMT</td>
<td>Phosphatidylethanolamine N-methyltransferase</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxalpyrophosphate</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RFC-1</td>
<td>Reduced folate carrier</td>
</tr>
<tr>
<td>SAH</td>
<td>S-Adenosylhomocysteine</td>
</tr>
<tr>
<td>SAH H</td>
<td>S-Adenosylhomocysteine hydrolase</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SHMT</td>
<td>Serine hydroxymethyltransferase</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofolate</td>
</tr>
<tr>
<td>TS</td>
<td>Thymidylate synthase</td>
</tr>
<tr>
<td>ZDF</td>
<td>Zucker diabetic fatty</td>
</tr>
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</table>
Normal folate, homocysteine, and methyl group metabolism is critical for maintaining optimum health, and perturbations in these mechanisms may lead to a number of pathological conditions such as diabetes, cardiovascular disease, neural tube defects, and cancer. Hyperhomocysteinemia is a condition that occurs when blood plasma concentrations of homocysteine exceed 15μM and has been implicated as an independent risk factor in cardiovascular disease. Specifically, populations with genetic polymorphisms in folate-dependent remethylation or poor B-vitamin status are at risk for this condition. This study was conducted in order to determine the influence of exercise on homocysteine and methyl group metabolism in a folate-deficient and -sufficient mouse model. Elevated homocysteine concentrations observed in the folate-deficient sedentary group were reduced by more than 2-fold with the addition of both forced treadmill and free access wheel exercise (p < 0.001). Similar results were observed in the folate-sufficient control diet with treadmill and wheel exercised mice having about 50% lower homocysteine concentrations than their sedentary counterparts (p < 0.001). Increased renal homocysteine remethylation through the enzyme betaine-homocysteine S-methyltransferase (BHMT) was observed in wheel exercised mice suggesting an increasingly important role in kidney homocysteine metabolism with exercise (p < 0.05). To our knowledge, this is the first study showing the attenuation of hyperhomocysteinemia in a model known to experience perturbed homocysteine metabolism. Findings from this study will be useful in determining the influence exercise has in other populations of altered homocysteine metabolism such as diabetics, vegans, and individuals with genetic polymorphisms.
CHAPTER 1 – GENERAL INTRODUCTION

Thesis Organization

This thesis is divided into four chapters including a general introduction, review of the literature, manuscript prepared for publication, and general conclusions. Chapter 1 includes an introduction on the significance of research in this area with a description of research questions. An extensive review of the literature will be found in Chapter 2 exploring folate, methyl group, and homocysteine metabolism and the importance of those mechanisms in the author’s original research. Chapter 3 will explain the impact exercise has on homocysteine and methyl group metabolism in a folate-deficient mouse model. General conclusions including ideas for future research will be included in Chapter 4 and a list of cited literature used throughout the thesis will be included at the end.

Description of the Research Questions

Folate, methyl group, and homocysteine metabolism are interrelated mechanisms that are required in a number of cellular processes including methylation of nucleic acids, proteins, and lipids. Perturbations in these pathways have been implicated in a number of disease states such as cardiovascular disease, diabetes, cancer, and neural tube defects. The search for therapeutic techniques aimed at normalizing these interconnected pathways is a major reason for conducting the present study.

The current literature regarding the influence of exercise on homocysteine, methyl group, and homocysteine metabolism is fairly controversial with a number of studies claiming an increase in homocysteine with exercise whereas others found a decrease or no
change at all (1-9). Moreover, most studies utilize the human model, which limits the use of invasive techniques for analyzing pertinent enzymes associated with methyl group and homocysteine metabolism in tissues such as the liver and kidney. Moreover, poor dietary records, such as ignoring B-vitamin status, and a variety of exercise modes among studies make it difficult to draw definitive conclusions. Therefore, we chose to utilize a folate-deficient mouse model, which possess a dietary status previously shown to display characteristics of hyperhomocysteinemia, so we could monitor food intake and exercise patterns (10, 11). In addition to collecting blood plasma for homocysteine concentration determination, we would also be able to further analyze enzymes important to homocysteine metabolism in both the liver and kidney.

The benefit of exercise is well established in the literature specifically with its favorable effects on the cardiovascular system; however, specific mechanisms regarding the benefits of exercise are not entirely clear (12, 13). Although controversial, hyperhomocysteinemia has been moderately accepted as an independent risk factor for cardiovascular disease and thus warrants further investigation concerning mechanisms to reduce homocysteine concentrations (14). The mouse model for this study is representative of populations prone to elevated homocysteine concentrations including individuals with genetic polymorphisms, such as in the enzyme methylenetetrahydrofolate reductase (MTHFR), vegans with poor vitamin status, and diabetics. Therefore, the goal of this study was to determine the influence of exercise on homocysteine metabolism in a folate-deficient mouse model. Results from this study can be further utilized in establishing the impact of exercise on other animal models of hyperhomocysteinemia.
CHAPTER 2 – REVIEW OF THE LITERATURE

Folate Structure and Function

Folate, a generic term used for the collection of folic acid derivatives and also referred to as vitamin B<sub>9</sub>, is required for a number of essential biochemical reactions in metabolism to sustain life (15). The physical structure of folate contains 3 main components, including a pterin ring, a <i>p</i>-aminobenzoic acid moiety, and a polyglutamate chain ranging from 1 to 14 glutamate amino acids (Figure 2.1) (15, 16). Complete reduction of the pyrazine ring of the pterin constituent renders the folic acid molecule into its coenzymatically active tetrahydro form, known as tetrahydrofolate (THF), allowing for the addition of 1 carbon moieties at the N-5 and/or N-10 position for a number of biological reactions known as one-carbon metabolism (16, 17). Major sources of methyl groups available for THF activation within the cell are derived from formate, serine, histidine, and purines (18). Moreover, compartmentalization among the cytosol, nucleus, and mitochondria within the

![Figure 2.1: Basic structure of the folate molecule (modified from 43)](image-url)
cell allows for strict control of one-carbon metabolism (18). One carbon units exist in many oxidation states ranging from the most reduced methyl group to the most oxidized formimino group, which will dictate its function within the cell (Figure 2.2) (15, 16).

As a vitamin and cofactor for numerous enzymes, folate must be acquired through the diet as humans are unable to synthesize it. However, gut bacteria can synthesize folate, which contributes to folate availability in the body (19). Polyglutamate forms of folate attained through the diet must be hydrolyzed into monoglutamate forms via folate hydrolase enzymes for absorption across the intestinal mucosa (16). Moreover, a folate-deficient state upregulates hydrolase enzymes to promote and enhance intestinal absorption (16).

Unfortunately, the bioavailability of folate from natural food sources is low due to the high levels of polyglutamate folate. This decrease in bioavailability may be due to evolutionary factors to limit folate concentrations in the body. However, folic acid provided in its synthetic form either as a supplement or through fortification has been previously shown to be highly bioavailable (20). Once in the monoglutamate form, folate is absorbed through the enterocyte via passive diffusion at high concentrations and through carrier protein encoded by the reduced folate carrier (RFC-1) gene in unsaturated, neutral pH conditions (19, 21, 22). As the name of the transport protein implies, folate is absorbed in its most reduced THF form.

<table>
<thead>
<tr>
<th>One carbon substituent</th>
<th>Position</th>
<th>Oxidation state</th>
</tr>
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<tbody>
<tr>
<td>Methyl</td>
<td>—CH₃</td>
<td>Methanol</td>
</tr>
<tr>
<td>Methylene</td>
<td>—CH₂</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Methenyl</td>
<td>—CH=</td>
<td>Formate</td>
</tr>
<tr>
<td>Formyl</td>
<td>—CHO</td>
<td>Formate</td>
</tr>
<tr>
<td>Formimino</td>
<td>HN=CH=</td>
<td>Formate</td>
</tr>
</tbody>
</table>

**Figure 2.2:** Folate constituents (modified from 16)
and is transported throughout the body as 5-methyltetrahydrofolate (5-CH$_3$-THF), which is primarily attached to albumin (22, 23). Once the 5-CH$_3$-THF reaches its cellular destination, the monoglutamate transport form is quickly altered to a polyglutamate form (5-CH$_3$-THF$_n$) via the enzyme folypolyglutamate synthase in order to prevent folate from diffusing from the cell (23, 24). In the cell, 5-CH$_3$-THF$_n$ acts in its coenzymatic role as a one-carbon donor and in its unmethylated form, it can act as a one-carbon acceptor to continue methyl group metabolism (23).

**Folate and One-Carbon Metabolism**

The main functions of folate within the cell are as a coenzyme and carrier of one carbon groups for both amino acid metabolism, including the regeneration of methionine from homocysteine, and nucleotide biosynthesis, such as purine and thymidylate synthesis (19). Moreover, the three major cycles requiring folate for one-carbon metabolism is comprised of the methionine, purine, and thymidylate cycles (22). Another important mechanism that provides additional methyl groups for folate is the interconversion of serine to glycine to generate 5,10-methylenetetrahydrofolate (5,10-CH$_2$-THF), an important derivative linking the previously mentioned cycles (22, 25). Serine hydroxymethyltransferase (SHMT) catalyzes the reversible reaction of serine to glycine, with the vitamin B$_6$ coenzyme pyridoxal-pyrophosphate (PLP), and has activity in both the cytoplasm and mitochondria via two isozymes (18). This interconversion of the nonessential amino acid serine to glycine may provide the most significant portion of methyl groups available to the cell and thus serves as an essential reaction to maintain the one-carbon pool (25).
Thymidylate synthesis requires the 5,10-CH\textsubscript{2}-THF coenzymatic form of folate for the irreversible synthesis of deoxythymidine-5-monophosphate (dTMP) from deoxyuridine-5-monophosphate (dUMP) by the enzyme thymidylate synthase (TS). dTMP can then be used for DNA synthesis and the oxidized folate, dihydrofolate (DHF), can be reactivated to the THF form by the enzyme dihydrofolate reductase (DHFR) and remethylated for further one-carbon reactions. Moreover, 5,10-CH\textsubscript{2}-THF can also be oxidized to 10-formyl-THF through a series of reactions for purine synthesis for nucleic acid synthesis (26).

Although nucleotide biosynthesis requires a significant amount of one-carbon groups from folate, methionine synthesis also utilizes a considerable number in the form of methyl groups. As a more intricate set of pathways and mechanisms, the methionine cycle involves not only a folate dependent remethylation pathway via the most reduced 5-CH\textsubscript{3}-THF form, it also contains a folate-independent remethylation, transsulfuration, and transmethylation pathway to carry out various functions in the cell collectively known as methyl group metabolism (22, 27).

**Methyl Group Metabolism**

Methionine plays a central role in methyl group metabolism as it provides the methyl group in the activated \textit{S}-adenosylmethionine (SAM) form for a number of constituents such as nucleic acids, lipids, and proteins (28). In order to maintain methyl group homeostasis within the cell to exist, sufficient regeneration of methionine from homocysteine, a metabolite in methyl group metabolism, and catabolism of homocysteine is vital for normal cell functioning. Dietary methionine also plays an important role in providing sufficient
substrate for transmethylation reactions in addition to a number of B-vitamins acting as coenzymes for both remethylation and transsulfuration reactions (Figure 2.3).

**Transmethylation**

As an essential amino acid, methionine participates in reactions collectively termed transmethylation through the substrate SAM, which is considered a key methyl group donor in nearly all biological reactions within the cell (27, 29). Although transmethylation reactions occur in many cells, the liver plays a large role in utilizing SAM in a number of biological reactions in addition to its synthesis and degradation (30, 31). Activation of SAM occurs via the enzyme methionine adenosyltransferase (MAT) utilizing the adenosyl moiety of

![Figure 2.3: Folate and one-carbon metabolism](image-url)
adenosine triphosphate (ATP) creating a substrate suitable for methyl group removal (32). The group of enzymes collectively called methyltransferases (MTs) remove the methyl group from SAM to an acceptor molecule forming S-Adenosylhomocysteine (SAH), which is then hydrolyzed to adenosine and homocysteine through the enzyme SAH hydrolase (SAH H), a reversible reaction (27, 30).

Although there are more than 100 discovered MTs, two of them utilize about 85% of methyl groups provided by SAM generating a significant amount of homocysteine in the cell. These MTs are phosphatidylethanolamine N-methyltransferase (PEMT) and guanidinoacetate N-methyltransferase (GAMT), which catalyze the formation of phosphatidylycholine (PC) from phosphatidylethanolamine (PE) and creatine from guanadinoacetate, respectively (31). After the synthesis of PC from PE via PEMT, PC can undergo further modification through phospholipases to create choline. This enzyme performs the only de novo biosynthesis of choline in mammals and utilizes 3 molecules of SAM per molecule of PC making it a large consumer of methyl groups (27, 33).

GAMT also consumes a significant amount of methyl groups in creatine biosynthesis. Guanadinoacetate, the substrate for GAMT, is synthesized by L-arginine-glycine amidinotransferase (AGAT), which creates ornithine and guanadinoacetate from arginine and glycine. The activity of AGAT occurs primarily in the kidney but the methylation reaction of GAMT occurs in the liver again emphasizing the importance of the liver in methylation reactions. The final product creatine is then utilized for energy metabolism, specifically in muscle and the nervous system (34). There have been discrepancies in the literature regarding the major consumer of methyl groups between PEMT and GAMT with one study
showing GAMT consuming 75% of available methyl groups with another more recent finding that PEMT consumed more than 2 to 3 times that of GAMT (35, 36). Although the high rate of methyl group consumption by these two enzymes illustrates an important aspect in methyl group metabolism, its influence on homocysteine concentrations may also be equally as important (37).

Another MT, glycine N-methyltransferase (GNMT), regulates the methyl group supply when they are abundant, such as excess dietary methionine, to prevent aberrant methylation within the cell. Glycine is the substrate for GNMT and disposes of the methyl group as sarcosine (38). However, in times of methyl group deficiencies or rapid cell division, such as in cancer, GNMT activity has been shown to be reduced to preserve methylation reactions (38, 39). GNMT is primarily expressed in the liver, representing 1-3% of the total cytosolic protein, but is also found in other tissues such as the kidney and pancreas (40). A perturbation in GNMT has previously been shown in various disease states including diabetes and cancer (40, 41).

**Folate-Dependent and -Independent Remethylation**

In the folate-dependent remethylation pathway, the fully reduced and methylated 5-CH$_3$-THF provides a methyl group to the non-protein forming amino acid homocysteine to form methionine (42). The irreversible reduction of 5, 10-CH$_2$-THF to form 5-CH$_3$-THF by the enzyme methylenetetrahydrofolate reductase (MTHFR) provides the vitamin B$_{12}$ dependent enzyme methionine synthase (MS) with substrate for methyl donation (43). As the reaction forming 5-CH$_3$-THF is irreversible, metabolic problems may arise if the substrate cannot be utilized within the cell. A vitamin B$_{12}$ deficiency prevents the transfer of methyl
groups to methionine, a disorder that will be discussed in a subsequent section. Upon
donation of its methyl group to methionine, THF can again acquire a methyl group from a
donor such as serine for another cycle of remethylation (44).

In addition to the folate-dependent pathway, methionine can be derived through a
folate independent pathway utilizing the enzyme betaine-homocysteine S-methyltransferase
(BHMT). BHMT removes the methyl group from betaine, or trimethylglycine, to remethylate
homocysteine and regenerate methionine and create dimethylglycine (DMG) (45). While
folate-dependent remethylation occurs in all tissues, folate-independent activity is limited to
kidney and liver tissue (42). Most betaine available to cells comes from the diet, however, the
nutrient choline can also be oxidized to provide betaine for methyl group metabolism (46).
Choline is also essential in the diet but can be derived from a methyl group expensive
transmethylation reaction in the cell, which was discussed previously (45).

**Transsulfuration**

The last mechanism in methyl group metabolism utilized to aid in the reduction of
homocysteine and synthesis of cysteine in the cell is the transsulfuration pathway (47). The
vitamin B₆ dependent enzyme cystathionine beta-synthase (CBS) condenses homocysteine
with the amino acid serine resulting in cystathionine. Further modification occurs when
another vitamin B₆ dependent enzyme cystathionine γ-lyase (CGL) cleaves cystathionine into
cysteine and α-ketobutyrate, a non-essential amino acid and key citric acid derivative
respectively (48). Although cysteine can be acquired through the diet, its de novo synthesis
can only occur through these set of reactions in animals and enzymatic dysfunction with
these enzymes may be detrimental to health (47). Likewise, the transsulfuration pathway
represents the only catabolic process for the removal of excess methionine and homocysteine in the cell and perturbations in enzymatic function could also be damaging to the cell. Cysteine, a final product of transsulfuration, can be further utilized in the cell to form glutathione, an intracellular redox buffer, in the glutathione synthesis pathway (48).

**Regulation of Methyl Group Metabolism**

Strict control of methyl group metabolism to limit hyper or hypomethylation of cellular proteins, lipids, or nucleic acids is imperative to maintain proper homeostasis within the cell. Aberrant methylation may be detrimental to the cell and prolonged perturbed methylation patterns may lead to disease. Therefore, allosteric and hormonal regulation of enzymes, in addition to nutrient availability, help normalize cellular methyl group metabolism.

**Allosteric Regulation**

As a significant source of methyl groups, the availability of dietary methionine can influence transmethylation in addition to the remethylation of homocysteine within a cell. It has previously been shown that in a diet supplying half the basal requirements of methionine, the reutilization of homocysteine through remethylation occurred more frequently rather than its removal through the catabolic transsulfuration pathway. However, when ample amounts of methionine were added to the diet, decreased remethylation and increased catabolism occurred (36). Moreover, other studies assessing the influence of methyl donors such as choline and betaine during a methionine deficient state show an induction of BHMT mRNA to aid in remethylation of homocysteine to sustain methyl group metabolism (49). GNMT is
also influenced by dietary methionine and in excess, GNMT activity increases potentially limiting methylation in the cell (38).

A key component in the allosteric regulation of methyl group metabolism is intracellular concentrations of SAM, especially in the liver (50). Moreover, the SAM:SAH ratio is an indication of transmethylation potential within the cell and the allosteric control associated with this ratio may be perturbed in certain pathologies (28). An important regulatory mechanism with a high ratio is the inhibition of MTHFR to prevent the irreversible formation of 5-CH$_3$-THF (51) (Figure 2.4). This inhibits the potential for

![Figure 2.4: Allosteric and hormonal control of methyl group metabolism](image)

**Glucocorticoids:** ↑ GNMT, MAT, CBS, BHMT

**Insulin:** ↓ CBS, BHMT

**Glucagon:** ↑ CBS

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hypermethylation in the cell by limiting the substrate 5-CH₃-THF for remethylating homocysteine. In addition, the high SAM:SAH ratio increases the catabolic activity of CBS, which decreases the concentration of homocysteine and thus reduces remethylation to methionine (48). BHMT is also regulated by SAM and when concentrations are elevated, BHMT is allosterically down-regulated to limit methyl group accumulation within the cell (52).

Furthermore, control of GNMT within the cell aids in the prevention of increased and aberrant methylation. 5-CH₃-THF can allosterically inhibit GNMT and when its concentrations are decreased with reduced MTHFR activity, GNMT activity increases to dispose of excess methyl groups as sarcosine (38, 39). Limiting hypermethylation within the cell prevents the downregulation of certain genes in addition to controlling protein activity. However, when SAM concentrations decrease and reduce methylation potential within the cell, MTHFR activity increases which in turn raises 5-CH₃-THF concentrations and allosterically prevents GNMT activity. It has also been presented that GNMT activity increases in its phosphorylated state and the binding of 5-CH₃-THF also prevents phosphorylation leading to reduced GNMT activity (53).

**Hormonal Regulation**

In addition to allosteric control, hormones have been demonstrated to influence methyl group metabolism. With the incorporation of retinoids in the diet, GNMT abundance and activity increased, potentially leading to reduced SAM dependent reactions. Moreover, increased hepatic GNMT activity from retinoid treatment resulted in the decrease of methyl group availability leading to hypomethylation in DNA (54, 55). Glucocorticoids, such as the
synthetic cortisol derivative dexamethasone, display similar hormonal regulation as retinoids and can increase the activity and abundance of GNMT (56). Furthermore, it has been shown with increased glucocorticoid concentrations, such as in times of high stress, MAT is up-regulated promoting the formation of SAM and increasing methylation potential within the cell (57). Additionally, CBS and BHMT are induced by glucocorticoids; however, insulin inhibited this induction suggesting its important role in regulating the transsulfuration and folate-independent pathways. Also, glucagon was shown to induce CBS promoting increased need of glucose precursors for gluconeogenesis provided in the transsulfuration pathway (58-60).

Recent studies have also shown that thyroid hormone plays a critical role in methyl group metabolism. In hypothyroidism, a decrease in MTHFR activity was observed, which would lead to an increase in GNMT activity from reduced 5-CH₃-THF concentrations (61, 62). Additionally, growth hormone may be important in promoting glutathione degradation, a product in the transsulfuration pathway, in addition to down-regulating MAT and GNMT activity reducing substrate availability for glutathione production. Reducing glutathione concentrations in the cell may decrease cell viability as glutathione aids in reducing oxidant damage (63).

**DNA Methylation and Epigenetics**

As the universal cellular methyl group donor, SAM is a key substrate for DNA methylation, an important mechanism in the regulation of gene expression. As all cells of multi-cellular organisms contain the same DNA, controlling gene expression is vital for cell differentiation and development (64). The term epigenetics means over or above the genome
but more specifically, it has been defined as “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence” (65). Perturbations in cellular methylation, such as nutritional deficiencies in methyl-supplying nutrients like methionine, may aid in the progression of disease (66).

Specific sites of DNA methylation occur on the 5’position of cytosine bases creating a 5-methyl-cytosine. Mammalian methylation has previously been shown to only occur on cytosine residues located next to a guanosine residue, which is called a CpG where the p stands for the phosphodiester bond linking the two (64). The regulatory role of methylation at CpG sites prevents or promotes the binding of transcription proteins through conformational changes in DNA structure. Specifically, a methylated CpG group prevents the binding of transcriptional proteins whereas an unmethylated one promotes protein binding (67).

Previous work has shown that about 5% of all cytosines in DNA are methylated and that 70-80% of global CpG sites, which are typically located in untranslated intron regions of genes and serve as a structural role to the genome, are methylated. The 20% of CpG sites that remain unmethylated are called CpG islands and are frequently found in promoter regions of genes (66).

DNA methyltransferases (DNMTs) are the enzymes responsible for methylating CpG sites using SAM as the methyl group donor. There are 4 major DNMTs including DNMT1, DNMT2, DNMT3a, and DNMT3b (64). DNMT 1 is responsible for methylating newly formed daughter strands of DNA of replicating cells to maintain similar methylation patterns of the parent cell (64, 68). Although its name suggests its activity towards DNA, DNMT2 has similar physical characteristics of DNMTs but has been shown to methylate RNA instead of
DNA (69). DNMT3a and DNMT3b exhibit similar functioning and participate in *de novo* DNA methylation within the cell, specifically unmethylated CpG sites (70). Moreover, DNMT3a and DNMT3b assist DNMT1 in proper methylation of the new daughter strands of DNA (64). In addition to direct methylation of DNA, proteins associated with DNA, called histones, can also be methylated to control gene expression. DNA wraps around histones, which compacts DNA and limits the accessibility of transcription machinery (71). Methylation of lysine tails located on histones both prevents and promotes transcription by closing or opening transcription binding sites, respectively, depending on location and number of methyl groups present on the tail (71). Moreover, specific arginine amino acids located on histones can also be methylated leading to increased gene expression (71).

The supply of methyl groups to DNA via SAM-dependent reactions is directly related to the availability of lipotropes within the cell. Deficiencies in lipotrope nutrients, which are ones that contain methyl groups like methionine, folate, and choline, may lead to perturbations in DNA methylation (72). More specifically, depletion of methyl groups may lead to hypomethylated DNA strands increasing the availability of promoter sites for gene transcription in addition to reducing DNA integrity leading to potential strand breaks. Extensive hypomethylation may be detrimental to the cell by increasing the expression of oncogenes promoting the development of cancer (73). Similarly, hypermethylation may silence tumor suppressor genes enhancing cancer development.

**Perturbations in Methyl Group Metabolism and Disease**

From a dietary standpoint, an adequate amino acid and B-vitamin profile are imperative for proper methyl group metabolism. Perturbations in dietary intake may
influence one-carbon metabolism and contribute to disease development. Moreover, genetic polymorphisms and preexisting diseases may also lead to alterations in methyl group metabolism independent of dietary status. As with any pathological condition, treatment options may be available to prevent and/or limit changes in one-carbon metabolism.

**Hyperhomocysteinemia**

The sulfur-containing amino acid homocysteine is created from the demethylation of methionine through the transmethylation of SAM (74). Homocysteine is also considered a non-protein forming amino acid and high concentrations in the bloodstream, termed hyperhomocysteinemia, may be detrimental to health (74, 75). Healthy concentrations of homocysteine in the bloodstream are between 5 and 10 micromoles per liter (µmol/L). However, hyperhomocysteinemia is categorized as having a concentration greater than 15 µmol/L, including 15 to 30, 30 to 100, and greater than 100 µmol/L for mild, intermediate, and severe hyperhomocysteinemia, respectively (76). Elevated homocysteine concentrations in the bloodstream can result from perturbations in transsulfuration, remethylation, and transmethylation mechanisms (77).

The transsulfuration pathway results in the irreversible catabolism of homocysteine and deficiencies in CBS activity or vitamin B₆ may hinder this mechanism (77). A mutation in the CBS enzyme is considered one of the most common causes of hyperhomocysteinemia, which may increase disease risk including vascular diseases and mental retardation (47). For instance, the 833T → C allele changes the function of CBS by limiting the binding potential of the enzyme to vitamin B₆ (78). This also exemplifies the importance of vitamin B₆ in the catabolism of homocysteine as it is an essential coenzyme for both CBS and CGL activity
Moreover, previous studies show that supplementation with vitamin B₆ may be beneficial in reducing homocysteine concentrations in healthy individuals (80). Along with the catabolism of homocysteine, the remethylation reactions also contribute to its reduction.

One of the major and most common deficiencies in folate-dependent remethylation of homocysteine occurs in the genetic mutation of MTHFR, which catalyzes the irreversible reduction of 5,10-CH₂-THF to 5-CH₃-THF. The most common polymorphism is the cytosine to thymine base pair switch that occurs at position 677, which changes the corresponding amino acid from a valine to an alanine (81). This single mutation decreases the stability of the enzyme when compared to other alleles (82). Additionally, the occurrence rate of this genetic polymorphism occurs in about 12% of the population who are homozygous with the T/T mutation whereas the heterozygous mutation C/T occurs in approximately 50% of the population (81). Another potential site of altered folate-dependent remethylation occurs with the enzyme MS in addition to its coenzyme vitamin B₁₂ or cobalamin.

One polymorphism of MS is the conversion of aspartic acid to glycine at the 2756 base pair from an A to G transformation. This mutation was shown to influence the binding of coenzyme B₁₂ and resulting in deficient catalytic activity in the remethylation of homocysteine to methionine (83). Furthermore, a lack of vitamin B₁₂ in the cell can also be detrimental to the remethylation of homocysteine due to its essential coenzymatic role. As the MTHFR reaction is irreversible, the idea of a methylfolate trap occurs as 5-CH₃-THF accumulates since it can only be utilized through this reaction within the cell (44). Without the transfer of a methyl group to methionine, free folate concentrations decrease limiting other folate-dependent reactions such as nucleotide biosynthesis (84). Moreover,
polyglutamation carried out by the enzyme folypolyglutamate synthase finds 5-CH$_3$-THF as
a poor substrate and thus the retention of folate within the cell through polyglutamation is
reduced (84). Furthermore, megaloblastic anemia is a disorder that arises typically from a
vitamin B$_{12}$ and/or folate deficiency causing malformation of red blood cells (85). The reason
for the development of large, immature blood cells seen in megaloblastic anemia is due to
insufficient substrate for RNA and DNA biosynthesis from inadequate levels in vitamin B$_{12}$
and/or folate (86).

Reduced transmethylation within the cell can also occur from hyperhomocysteinemia.
Since the conversion of SAH to homocysteine is a reversible reaction and exists in
equilibrium, increased concentrations of homocysteine will also increase SAH
concentrations. This creates an unfavorable situation as SAH acts as an allosteric inhibitor of
some methyltransferases limiting transmethylation reactions in the cell (87). In addition to
perturbations in transmethylation from feedback mechanisms of SAH, a reduction in both
folate-dependent and -independent remethylation of homocysteine may limit methionine
availability for SAM dependent reactions (42). Moreover, a mutation in GNMT may lead to
altered methyl group metabolism. As a tetramer, GNMT can exist in a number of different
conformations and if one subunit acquires a mutation, it may lead to a potentially inactive
enzyme (88). Since GNMT plays a regulatory role in modulating methyl group metabolism,
aberrant methylation may occur if the gene becomes defective.

**Cardiovascular Disease**

With increased homocysteine concentrations in the bloodstream, the risk of
developing disease is also elevated. More specifically, hyperhomocysteinemia has been
implicated in cardiovascular disease, such as sudden death, stroke, arterial disease, and thromboembolisms (74). Although the literature is fairly controversial regarding the impact of hyperhomocysteinemia on the cardiovascular system, a number of case-controlled studies have demonstrated that increases in homocysteine concentrations in plasma may increase the risk of cardiovascular related events by 2-fold (89). Arguments for the detrimental effects of hyperhomocysteinemia on the cardiovascular system are related to the damage of endothelial cells caused by oxidative stress and inflammation (90). Moreover, homocysteine was observed to exhibit similar properties in endothelial cell damage as hypercholesterolemia and hypertension resulting in endothelial dysfunction or limited relaxation of blood vessels (90). Oxidation of the vasodilator nitric oxide is one specific proposed mechanism regarding the detrimental effects of hyperhomocysteinemia (91). Since homocysteine contains a thiol group, it can become oxidized and form reactive oxygen species, which can then cause oxidative damage and limit the bioavailability of nitric oxide in the vasculature (92).

**Neural Tube Defects**

Along with the concern for cardiovascular disease and elevated homocysteine concentrations, neural tube defects (NTDs) have been associated with perturbed methyl group metabolism specifically regarding maternal folate status. During embryogenesis, the first four weeks are most critical for neurological development and if neurogenesis is hindered, the neural tube may not close leading to an NTD (93). Two major forms of NTDs are anencephaly, which usually results in a stillborn or in death shortly after delivery, and spina bifida, which leads to major disabilities (94). Recent studies have shown that folic acid may reduce the incidence of NTDs leading the Centers for Disease Control and Prevention
(CDC) to change their standards in food fortification requirements (95, 96). Along with a recommendation for women of reproductive age to consume 0.4 mg of folic acid and a folate-rich diet in 1992, the CDC made fortification of 140 μg per 100 g of cereal grain mandatory in 1998 leading to a 20-30% decrease in the prevalence of NTDs; yet, the current fortification requirement has received much scrutiny as it may not be high enough (95-97). Genetic variations such as single nucleotide polymorphisms (SNPs), perturbed cellular methylation, and folate receptor antibodies are some of the mechanisms explaining the importance of folate for the prevention of NTDs.

The MTHFR polymorphism, as described previously, leads to reduced cellular folate available for remethylation of homocysteine and may impact neurogenesis; however, it is still unclear how this polymorphism in the mother influences embryo development (98). Autoantibodies, which are immune proteins that target one’s own cells, may also influence the availability of folate within the cell. For instance, an autoantibody for the folate-receptor membrane protein was discovered in mothers who previously experienced a pregnancy with an NTD. Although the mechanism regarding autoantibody formation and activity is not specifically known, investigators hypothesize it may contribute to a combination of reproductive tissue injury, proteolysis, and genetic mutations leading to the formation of autoantibodies against folate receptors (99). Moreover, DNA methylation is thought to influence gene expression during embryogenesis and a lack of methyl groups from inadequate folate consumption may influence NTD development (93).
Diabetes

Another disease known to influence homocysteine and methyl group metabolism is diabetes. Currently, a clear relationship between diabetes and changes in homocysteine concentrations does not exist; however, recent studies have shown that increases in homocysteine may be related to disease progression, such as in cases of nephropathy (29). Moreover, a definitive conclusion regarding the influence of homocysteine on insulin resistance is inconclusive with reports of positive, negative, and no correlation between the two (100-102). In a streptozotocin (STZ) induced type I diabetic rat model, plasma homocysteine concentrations were about 40% lower than their control counterparts. However, the addition of insulin to this model prevented the drop in homocysteine concentrations when compared to the control group. The decrease in homocysteine concentrations observed in the type I diabetic rat model may have been due to increased transsulfuration enzymes as insulin treatment normalized their activity (58). Also, DNA hypomethylation patterns were observed in the same STZ induced rat model suggesting that aberrant methylation of DNA in a type I diabetic may lead to altered gene expression (103).

In type II diabetes, the change in insulin response during disease progression may be related to changes in homocysteine and methyl group metabolism. A typical model used in type II diabetic research is the Zucker diabetic fatty (ZDF) rat model, which displays similar metabolic perturbations seen in type II diabetes through the development of obesity (104). Similarly to type I diabetic rats, type II ZDF rats in an insulin resistant state display decreased homocysteine concentrations and increased activity of the CBS and CGL transsulfuration enzymes. However, activity of the remethylation enzyme BHMT is also
elevated in ZDF rats (105). Moreover, nephropathy in severe cases of diabetes leads to increases in homocysteine concentrations and may be attributed to reduced glomerular filtration in the kidneys (106, 107). Also, cardiovascular disease risks are greater in the diabetic population and thus maintaining proper homocysteine and methyl group metabolism may limit the progression of vascular problems in this population (108).

**Cancer**

Perturbations in homocysteine and methyl group metabolism have also been implicated in carcinogenesis in addition to the treatment and prevention of cancer. As stated previously, folate plays a major role in the formation of dTMP from the precursor dUMP. More specifically, 5,10-CH₂-THF is the one-carbon donor in this reaction and is considered essential for DNA synthesis (109). Thus, a folate deficiency may lead to improper DNA synthesis with the loss of thymine for base pairing aiding the development of cancer. Moreover, as folate participates in a number of one-carbon reactions, such as methionine and purine synthesis, a folate deficiency in addition to perturbed one-carbon enzyme function may exhaust folate sources within the cell (109). Furthermore, a deficiency in dTMP promotes the incorporation of uracil into DNA leading to double strand breaks, chromosomal damage, or genomic alterations leading to carcinogenesis. However, proper folate intake limited the incorporation of uracil into DNA in a human population, which may prevent mutations in DNA and cancer development (110). Moreover, it has been shown that decreased folate intake may negatively influence tumor protein 53 (p53), an important protein involved with cell cycle and cell signal regulation during cell growth and division (111). A recent report illustrated that the expression and structural integrity of p53 may be
influenced by inadequate folate intake potentially leading to the development of neoplasms (112).

Although adequate folate intake may be beneficial in the prevention of cancer, it may also prove to be detrimental in the progression of neoplasms. In the case of a preexisting tumor or cancer cell, increased cellular concentrations of folate may promote cancer cell development leading to increased proliferation and growth (113). An explanation of this disease progression may be due to the significant amount of folate cancerous cells need to carry out DNA synthesis (114). Additionally, folic acid fortification of food and supplementation with multivitamins may also influence this disease progression as folic acid is not the naturally occurring form of the vitamin found in food and is readily absorbable. Since folate is converted to its active form through the intestinal wall, poor bioavailability of folate may act as a regulatory mechanism to prevent over absorption (114). Therefore, timing of supplementation in terms of cancer progression may be a factor when considering dietary recommendations.

With the notion that folate may contribute to carcinogenesis, antifolates were derived in order to disrupt the formation of DNA synthesis through the inhibition of thymidylate and purine biosynthesis (115). Specifically, antifolates, such as Methotrexate (MTX) or Nolatrexed, have a wide range of action including the inhibition of DHFR or TS, respectively. MTX can enter the cell through the same RFC-1 as reduced folates and once inside the cell, MTX can bind tightly to DHFR rendering it inactive through competitive inhibition. Moreover, MTX is structurally different than folate in that the hydroxyl group located on the fourth carbon on the pteridine ring on THF is replaced with an amino group
changing substrate binding properties (116) (Figure 2.5). Thus the irreversible binding of DHFR to MTX prevents the enzymatic reduction of DHF in the cell by this enzyme, which is necessary for purine and thymidylate synthesis. A newer antifolate Nolatrexed inhibits TS leading to the reduction of thymidine production from dUMP limiting DNA synthesis (116). As polyglutamation and an RFC-1 is required by many antifolates for successful inhibition, such as MTX, Nolatrexed doesn’t require either, potentially leading to greater drug availability and reduced drug resistance (117).

In addition to folate contributing to carcinogenesis, epigenetic changes also influence cancer development. For instance, promoter regions of tumor suppressor genes become heavily methylated leading to decreased expression of genes vital to cellular functions such as cell cycle control and DNA repair (118). However, global DNA hypomethylation occurs in many types of breast cancer leading to increased transposable elements, which then insert

![Structural comparison of tetrahydrofolate and Methotrexate](https://example.com/figure25.png)

**Figure 2.5:** Structural comparison of tetrahydrofolate and Methotrexate (Modified from 116)
into other places in the genome mutating other genes (119). Moreover, perturbed histone modification also promotes carcinogenesis. Specifically, the loss of acetylation and methylation on specific lysine residues of histone H4 may be a significant marker of cancer development and may aid in earlier diagnosis (120). Interestingly, using epigenetic mechanisms for chemotherapy, such as the reactivation of tumor suppressor genes through hypomethylating mechanisms, could potentially be used for cancer treatment (121).

**Impact of Exercise on Methyl Group and Homocysteine Metabolism**

The benefit of physical activity on human health across the lifespan, especially regarding the cardiovascular system, is well established in the literature (12, 13). Although the specific mechanism of improved vascular function with exercise is not completely understood, changes in arterial stiffness and vascular endothelial cells with physical activity may give rise to cardiovascular disease prevention (13). As stated previously, the contribution of homocysteine on vascular dysfunction is controversial and has only been moderately accepted as an independent risk factor for cardiovascular disease (14). Moreover, the influence of exercise on plasma homocysteine concentrations is not well established due to significant discrepancies in the literature (122).

Two major reasons for the inconsistency in the literature regarding the influence of exercise on homocysteine concentrations are the variety of intensities and durations among studies. With a single training bout, investigators found an increase, decrease, and no change in homocysteine concentrations after exercise when compared to pre-exercise concentrations (5, 6, 9). Moreover, the influence of chronic exercise on homocysteine concentrations in trained athletes is also inconclusive as studies displayed similar results with increased,
decreased, and no differences in concentration (1, 2, 7). Furthermore, the addition of chronic exercise training for sedentary individuals was inconsistent resulting in elevated, reduced, and no change in homocysteine concentrations (3, 4, 8). The type of exercise and sex of the subjects also varied among the previously mentioned studies. Diet records were also not consistently controlled for; therefore, intake of pertinent B-vitamins required for methyl group metabolism was not accounted for. Moreover, these studies only used the human model increasing the error in exercise intensity reporting during testing and diet recall (123).

**Amino Acid Availability**

Mechanistically, the influence of physical activity on homocysteine concentrations can support both an increase and decrease based on protein and methyl group turnover. With exercise, increased methionine catabolism through homocysteine for cysteine synthesis or gluconeogenic precursors would lead to a reduction in homocysteine concentrations (123). Under conditions of physical exertion, especially during prolonged periods, amino acid catabolism increases to fulfill energy requirements for the body (124). Moreover, the mode of exercise may also influence methionine catabolism. For instance, weight lifting women had reduced concentrations of methionine in their bloodstream after a single bout of squat exercises (125). Also, high protein intake in combination with exercise may also influence amino acid availability in the bloodstream (126). A reduction in methionine concentrations from poor dietary intake and/or increased catabolism through exercise would promote increased methionine synthesis from homocysteine for protein formation assuming the individual has proper B-vitamin status for remethylation. Therefore, increased remethylation
of homocysteine due to changes in methionine requirements for protein synthesis during exercise should reduce homocysteine concentrations (123).

In addition to potential homocysteine reducing effects in the plasma through protein turnover, B-vitamin status may hinder this process and lead to increased homocysteine concentrations. PLP, the active form of vitamin B₆, is essential in catabolic reactions involving amino acids including homocysteine (123). During exercise, it has been shown that PLP levels increase in the bloodstream, potentially increasing the demand for PLP during amino acid catabolism in other tissues and its loss through excretion (123, 127, 128). Moreover, the decrease in PLP would also reduce the catabolism of homocysteine during exercise through the transsulfuration pathway due to its coenzymatic role (79).

**Increased Transmethylation and Remethylation**

Another mechanism influencing homocysteine concentrations in the bloodstream is the increase in methyl group donation especially during high intensity exercise. A transmethylation reaction utilizing as much as 75% of the methyl groups made available by SAM is the formation of creatine from guanidinoacetate through the enzyme GAMT (36). Creatine is located primarily in muscle and in its phosphorylated form functions by rephosphorylating adenosine diphosphate (ADP) to ATP thereby replenishing energy stores especially in short high intensity activities. After this transfer, creatine can then be reutilized for further reactions involving the rephosphorylation of ADP or excreted through the urine as creatinine (129). Thus, mode of exercise may be a significant factor in the formation of homocysteine due to increased GAMT activity during high intensity and short duration exercises that may rely more on methyl group metabolism than a longer duration and lower
intensity exercise (123). Interestingly, supplementation with creatine reduced homocysteine concentrations in a rat model suggesting the potential down-regulation of GAMT when creatine is present in the cell through feedback mechanisms (130). Another study found that subjects, after a bout of exercise, experienced increased endogenous plasma creatine levels leading to a decrease in reduced homocysteine, the form present right after synthesis from SAH, proposing a potential homocysteine reducing mechanism through feedback inhibition from elevated creatine concentrations in the body (131).

Choline, an important component in supplying methyl groups in homocysteine and methyl group metabolism, may also be influenced by physical activity (132). After absorption by the intestine, choline is either phosphorylated to PC or oxidized to betaine in cells such as hepatocytes (33). It has previously been shown that in a methyl rich environment from a methionine load, supplementing with betaine and PC normalized homocysteine concentrations; however, this reduction did not occur with folate supplementation (133). Therefore, the folate-independent enzyme BHMT may be utilized more readily in times of high methyl group demand in the liver such as during exercise (132). Although an increase in BHMT activity might be expected in the liver with exercise, other tissues may respond differently. For instance, the expression of BHMT in the colon of rats exposed to treadmill exercise was reduced, potentially limiting aberrant methylation, which is typically seen in colon cancer, by reducing methyl group supply (134).

Perturbed methyl group and homocysteine metabolism is linked to a number of diseases and characterizing changes in these mechanisms may give rise to therapies to limit or prevent disease progression. Moreover, understanding how exercise influences
homocysteine concentrations may be important not only in healthy populations but also ones subject to perturbed methyl group metabolism and homocysteine metabolism, such as diabetics and individuals with polymorphisms. Therefore, well controlled studies including ones with special populations, such as those with the MTHFR polymorphism, are necessary to identify the potential benefits of exercise for normalizing one-carbon metabolism. Additionally, as specific mechanisms regarding the influence of exercise on the cardiovascular system are established, changes in homocysteine metabolism may be a contributing pathway in the prevention of cardiovascular disease with regular physical activity. Lastly, identifying the most efficient mode and duration of exercise may help tailor the recommendation for achieving optimal homocysteine and methyl group metabolism.
CHAPTER 3 – EXERCISE PREVENTS HYPERHOMOCYSTEINEMIA IN A FOLATE-DEFICIENT MOUSE MODEL

Abstract

Perturbations in homocysteine and methyl group metabolism have been implicated in a number of disease states such as cardiovascular disease, cancer, and diabetes. Hyperhomocysteinemia occurs with elevated concentrations of homocysteine in the bloodstream, and has been indicated as an independent risk factor for progression of cardiovascular disease. Removal of homocysteine through remethylation or transsulfuration mechanisms is utilized in reducing homocysteine concentrations in the blood. The present study examined homocysteine concentrations and pertinent hepatic and renal methyl group enzymes in an outbred imprinting control region (ICR) mouse being fed a folate-deficient or -sufficient diet. Within each diet treatment, mice were further divided among three groups: sedentary, free-access wheel exercise, and forced treadmill exercise. Both treadmill and wheel exercised folate-deficient mice displayed greater than 2-fold reduction in homocysteine concentrations compared to their sedentary counterpart (p < 0.001). Moreover, when control diet mice were considered separately, plasma homocysteine concentrations in treadmill and wheel exercised mice were significantly lower than their sedentary counterpart (p < 0.05). To our knowledge, this is the first study demonstrating that exercise prevented dietary-induced hyperhomocysteinemia in a mouse model. The results from this study may serve as a platform for understanding the protective effects of exercise on pathologies associated with hyperhomocysteinemia.
Introduction

Maintaining optimal one-carbon metabolism is essential to promote healthy living. Perturbations in these pathways may have significant implications in a number of disease states including cancer, cardiovascular disease, neural tube defects, and cognitive disorders (135-138). Nutritional status and genetic mutations have profound effects on the interrelated mechanisms involving one-carbon metabolism and dietary interventions to maintain optimal methyl group, folate, and homocysteine metabolism, such as B-vitamin supplementation, may help reduce disease progression (139). Moreover, factors such as supplementation with soy isoflavones and increased physical activity are potential modulators of these pathways aimed specifically at reducing homocysteine concentrations (140, 141).

Hyperhomocysteinemia results with the accumulation of homocysteine in the bloodstream and has previously been shown to be an independent risk factor for the development of cardiovascular disease (142, 143).

Homocysteine formation results from methionine metabolism through the transmethylation pathway in which the active form of methionine, S-adenosylmethionine (SAM), donates a methyl group to a number of acceptor molecules, including proteins, lipids, and nucleic acids (28). Homocysteine is then removed via remethylation by folate-dependent or -independent mechanisms or irreversibly catabolized through the transsulfuration pathway. Folate-dependent remethylation not only relies on the B-vitamin folic acid to provide one-carbon units for the formation of methionine from homocysteine but also vitamin B_{12} as a coenzyme for methionine synthase (MS), which utilizes the substrate 5-methyltetrahydrofolate (5-CH_{3}-THF) as a methyl group supply (28). Moreover, folate-
independent remethylation of homocysteine utilizes the enzyme betaine-homocysteine S-methyltransferase (BHMT) to transfer a methyl group from betaine to homocysteine (45). Irreversible catabolism of homocysteine through the transsulfuration pathway is a vitamin B6-dependent reaction through the enzyme cystathionine β-synthase (CBS) which catalyzes the eventual formation of cysteine (47).

Transmethylation reactions are carried out by more than 100 methyltransferases (MTs) with about 85% of methyl groups being utilized by the enzymes phosphatidylethanolamine N-methyltransferase (PEMT) and guanidinoacetate N-methyltransferase (GAMT) (36, 144). PEMT utilizes three methyl groups from SAM to form a phosphatidylcholine (PC) molecule, which generates three homocysteine molecules. It has previously been shown in a PEMT knockout model, a lack of PEMT activity significantly reduced homocysteine concentrations demonstrating its important role in homocysteine production (145). Additionally, supplementation with creatine, the product of the GAMT reaction and another large consumer of methyl groups, resulted in a 27% decrease in homocysteine concentrations (130). Therefore, creatine supplementation leads to a feedback mechanism regulating its formation reducing methyl group consumption and homocysteine production (130).

Regulation of transmethylation is carried out by the enzyme glycine N-methyltransferase (GNMT), which is the most abundant methyltransferases in the liver and acts to optimize transmethylation reactions within the cell by disposing of excess methyl groups as sarcosine (39). Lack of GNMT activity in both a mouse knockout model and humans deficient in the enzyme have been previously examined and both models displayed
elevations in SAM concentrations and decreases in S-adenosylhomocysteine (SAH), a product of transmethylation reactions that is interconvertible with homocysteine based on product concentration (146, 147). Therefore, increases in the SAM:SAH ratio may increase methylation potential within the cell, which may lead to aberrant methylation of cell constituents. However, decreasing the ratio reduces transmethylation activity, potentially leading to hypomethylation of cellular components (148). Moreover, increased levels of SAH observed in a heterozygous CBS knockout model was directly correlated with increased homocysteine concentrations further signifying the importance of homocysteine removal in maintaining an optimal SAM:SAH ratio for transmethylation reactions and prevention of hyperhomocysteinemia (149).

Recently, a number of studies assessing the influence of exercise on homocysteine concentrations in humans are fairly ambiguous in regards to acute, chronic, or sedentary exercised individuals (1-9). A major issue that contributes to the discrepancies in human studies measuring homocysteine concentrations is the inadequacy of monitoring essential B-vitamins required for homocysteine metabolism in addition to subject training status, discrepancies in exercise intensity reporting, and variations in mode, intensity, and duration among studies (Husemoen, Thomsen et al. 2004; Joubert and Manore 2006).

Mechanistically, a reduction in homocysteine concentrations with exercise may be related to increased protein turnover. More specifically, it has been measured in both humans and rats that methionine concentration in plasma significantly increases during exercise and in humans, methionine concentration was shown to decrease below basal levels hours after exercise (150-153). This suggests that decreased plasma methionine after an exercise bout
may be due in part to increased muscle anabolism requiring methionine thus leading to reduced substrate for transmethylation reactions and homocysteine concentration (123, 153). On the contrary, exercise increases vitamin B₆ demand with increased muscle catabolism and excretion which may potentially elevate homocysteine concentrations (128).

The present study assessed the influence of both voluntary and involuntary exercise in a control and folate-deficient mouse model. The folate-deficient model was used to represent a population of individuals with folate absorption deficiencies, reduced folate intake, or nucleotide polymorphisms such as in the 5,10- methylenetetrahydrofolate reductase (MTHFR) gene, a key enzyme supplying 5-CH₃-THF for homocysteine remethylation (81). Previously, a folate deficiency has been shown to significantly elevate homocysteine concentrations in both human and rodent models and this study aimed at preventing its rise with the addition of exercise (10, 11). In the present study, we utilized an exercised folate-deficient mouse model for measuring both hepatic and renal enzyme function and expression involved with homocysteine and methyl group metabolism.

**Experimental Procedures**

**Animals and Diets**

Animal usage and care was carried out in accordance with Iowa State University Institutional Animal Care and Use Committee (IACUC). Thirty-two Female Imprinting Control Region (ICR) mice (Harlan, Indianapolis, IN) aged 9-10 weeks were initially assigned randomly to cages consisting of 2 or 3 in a 12-hour light:dark cycle room and were allowed access to food and water *ad libitum*. Mice were acclimated for 3 days on an AIN-
93G (Harlan Teklad, Indianapolis, IN) control diet containing 40.2% cornstarch, 39.3% dextrose, 10% casein, 4% mineral mix (AIN-93G-MX), 1% vitamin mix (AIN-93-VX), 5% corn oil, 0.2% 1.5 M choline bitartrate, and 0.3% L-methionine.

At day 4, mice were divided into the following 3 groups: sedentary (n=11), forced treadmill exercised (n=11), or free access wheel exercised (n=10). Wheel exercised mice were housed in individual cages in order for accurate distance calculation whereas the sedentary or treadmill exercised were housed in groups of 2 or 3. Wheel exercised mice had free access to their wheel 24 hours for 5 days a week for the duration of the study whereas treadmill exercised mice were forced to run 5 days a week. A final treadmill speed of 16-16.5 meters per minute for a total of 45 minutes was reached at 28 days, with 18 of those being exercise trained, and this speed and duration was used for the remainder of the study. At day 29, a folate-deficient diet was introduced and mice again were divided within their exercise treatment into 6 groups: control diet sedentary (n=5), forced treadmill exercised (n=5), and free access wheel exercised (n=5) and a folate-deficient diet sedentary (n=6), forced treadmill exercised (n=6), and free access wheel exercised (n=5).

On day 77, after 36 days of exercise training with diet treatment, mice were fasted for 12 hours and given an intraperitoneal injection of fresh ketamine (90 mg/kg body weight) and xylazine (10 mg/kg body weight) prior to necropsy. A cardiac puncture was performed with a heparinized syringe and whole blood samples were then centrifuged at 4000 x g for 6 minutes to separate the plasma fraction for homocysteine and glutathione concentration determination. Plasma samples were then frozen at -20°C until analysis. Liver tissue was collected and 0.5 gram portions were immediately homogenized in a 2 mL ice-cold solution
of 10 mM sodium phosphate of pH 7, 1 mM EDTA, 1 mM sodium azide, 0.25 M sucrose, and 0.1 M phenylmethylsulfonyl fluoride. Remaining liver tissue was immediately snap frozen in liquid nitrogen and stored at -80°C for future RNA analysis. Homogenized tissue was then centrifuged at 20,000 x g at 4°C for 30 minutes and the protein layer was removed, placed in 1.5 mL Eppendorf tubes, and also stored at -80°C for subsequent enzyme analysis. One kidney was homogenized in the same solution utilized for liver tissue in a 1:4 weight to volume ratio, centrifuged at 20,000 x g at 4°C for 30 minutes, and the protein isolate stored at -80°C for future enzyme analysis. The second kidney was snap frozen in liquid nitrogen and also stored at -80°C.

**Determination of Hepatic and Renal Protein Concentrations**

For protein concentration determination of both liver and kidney homogenates, the Pierce Bicinchoninic Acid (BCA) method was used with a protein assay kit (Thermo Scientific, Rockford, IL; Pierce Microplate BCA Protein Assay Kit). A standard curve was first established using a 2 mg/mL concentration of bovine serum albumin (BSA) on a 96 well plate. Each dilution in the BSA standard curve was measured in triplicate with concentrations of 2 mg/mL and to 0 mg/mL and a final volume of 17.5 μL in each well. Liver and kidney samples were then thawed completely on ice, vortexed thoroughly, and then 2.5 μL of each sample was transferred in triplicate to the 96 well plate. Fifteen μL of deionized water was then added to the plate to bring the volume to 17.5 μL equaling that of the standard curve. One hundred sixty μL of Pierce BCA reagents A and B in a 50:1 ratio, respectively, was added to each well and allowed to sit covered for 45 minutes prior to plate reading. Absorbance was then read at 562 nm and protein concentrations were analyzed.
Determination of Homocysteine and Glutathione Concentrations

Total homocysteine and glutathione concentrations were assessed using both High Performance Liquid Chromatography (HPLC) and fluorescence detection (154). Blood plasma samples were thawed and 100 μL of each sample was diluted with 200 μL deionized water for a final volume of 300 μL. 1 mM N-acetylcysteine was added to each sample as an internal standard and then incubated for 30 minutes at 4°C in a 10% vol/vol solution of tributylphosphine in dimethylformamide. Ice cold 10% trichloroacetic acid with 1 mM EDTA was added to stop the reaction and the samples were centrifuged at 4°C for 5 min at 1000 x g. The supernatant was collected and added to tubes containing 0.125 borate buffer with pH 9.5, 0.1% 4-fluoro-7-sulfobenzofurazan, and 1.55 M sodium hydroxide. Before injection into the HPLC machine, each sample was filtered using an aerodisc filter (0.22 μm) and transferred to new 1.5 mL tubes. Samples were then added (100 μL) into a μBondapak C₁₈ Radial-Pak column (Waters) for separation in a mobile phase containing 4% acetonitrile and 96% 0.1 M monobasic potassium phosphate buffer (pH 2.1). Chromatograph print outs were computer analyzed using the area under the curve method.

Glycine N-methyltransferase (GNMT) Activity

GNMT enzymatic activity was measured based on a previously established method with some modifications (155). The reaction mixture (100 μL) contained 2 mM glycine, 200 mM Tris buffer at pH of 9.0, 0.2 mM S-adenosyl-L-[methyl-³H] methionine, and 5 mM dithiothreitol. To initiate the reaction, protein aliquots of 250 μg for each sample were added to separate Eppendorf tubes in triplicate with a heat denatured sample serving as a blank. After a 30 minute incubation period at 25°C, 50 μL of 10% trichloroacetic acid was added to
each Eppendorf tube to stop the reaction. In order to remove excess radiolabeled substrate, 250 μL of an activated charcoal suspension (78 mg/mL) in 0.1 M acetic acid was added to each sample. The samples were then kept at 4°C for at least 20 minutes before centrifugation at 4°C for 5 minutes at 13,500 x g. Supernatant aliquots of 200 μL from each sample were then added to scintillation vials with 10 ml Scintiverse (Fisher Scientific, Fair Lawn, NJ) for radioactivity quantification through liquid scintillation counting.

**Methionine Synthase (MS) Activity**

Determination of methionine synthase (MS) activity was originally performed by Keating and others. (156). AG 1-X8 resin (Cl⁻ form, 100-200 mesh) was used for anion exchange. For resin prep, approximately 75 g was washed 3 times with approximately 900 mL of deionized water in order to clean the resin and remove impurities. The resin was then placed in 900 mL of 0.5 M HCl, swirled slightly, and allowed to sit for 90 minutes to prime the resin beads. The resin was washed 3 more times in 900 mL of deionized water followed by a 900 mL wash with 0.5 M sodium hydroxide. Next, the resin was washed again with 900 mL 3 times with deionized water followed by another acid wash of 1500 ml 0.5 M HCl. After a final rinse with 300 mL of deionized water, the resin was vacuum filtered and washed using a Buchner funnel and Whatman filter paper and then stored in deionized water at room temperature until use.

The reaction mix was prepared with 100 mM DL-homocysteine made fresh before use, 1.3 mM cyanocobalamin, 500 mM sodium phosphate buffer of pH 7.5, 10 mM S-adenosylmethionine, 82.4 mM 2-mercaptoethanol, 1 M dithiothreitol, 15 mM 5-CH₃-tetrahydrofolate, 15 mM 5-[¹⁴CH₃]-tetrahydrofolate, and deionized water. Hepatic mouse
protein (600 μg) was added to eppendorf tubes and a random sample was utilized as a heat denatured blank being exposed to 75°C for 10 minutes. Each sample received 100 μL of the reaction mix and incubated at 37°C for 1 hour and at the end of incubation, 800 μL of ice-cold deionized water was added to each sample to stop the reaction. Columns were prepped with approximately 1 mL of resin and each sample was added to the column to remove excess radiolabeled substrate with the effluent being collected in a scintillation vial. The resin was washed twice with 1 mL deionized water and the effluent was again collected in scintillation vials. The scintillation vials were then filled with 10 mL of Scintiverse and the radiolabeled product was measured using a liquid scintillation counter.

**Betaine Homocysteine S-methyltransferase (BHMT) Activity**

BHMT activity was previously analyzed and described by Garrow and performed with some modifications (157). Resin prep with 100 g of Dowex 1x4 (OH- form, 100-200 mesh) was washed with 1 L of 2.5 M HCl, stirred, and allowed to settle. The resin was then washed twice with 2 L of deionized water and then with 1 L of 500 mM NaOH. A Buchner funnel with Whatman filter paper was then used to wash the resin with 2 L of 1 M NaOH and a final wash with about 12 L deionized water. The resin was stored at 4°C in a 50% slurry with deionized water until use. The reaction mix contained the following: 50 mM [14CH₃]-betaine, 100 mM DL-homocysteine, 500 mM Tris (pH 7.5), 5% BSA, 10% 2-mercaptoethanol solution, and deionized water. For hepatic tissue samples, 40 μg of protein was used and 100 μg was used for renal tissue. Protein samples were added in duplicate to the reaction mixture in glass tubes, placed in ice, and covered with rubber stoppers. Samples were incubated at 37°C for 1 hour, immediately placed back on ice, and 2.5 mL of ice-cold
deionized water was added to stop the reaction. Approximately 1 mL of resin was added to each column and the sample was loaded. The column was then washed 3 times with 5 mL of water and a final wash with 3 mL of 1.5 M HCl was collected in a scintillation vial. After the addition of 10 mL of Scintiverse, radioactivity measurement was done by a liquid scintillation counter.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

RNA was first isolated from 50-100 mg of liver tissue using liquid nitrogen to snap freeze the tissue and then homogenized using the mortar and pistol method. The homogenized tissue was then transferred to an Eppendorf tube containing 1 mL of Trizol (Invitrogen, Carlsbad, CA) and then vortexed and stored in at -80°C overnight to facilitate RNA extraction. Samples were then thawed, vortexed, and centrifuged at 12,000 x g for 10 minutes at 4°C in order to remove unwanted debris. Supernatants were transferred to a new Eppendorf tube and allowed to sit at room temperature to dissociate nuclease proteins. A molecular grade chloroform wash (200 μL) was added to each tube, vortexed gently, incubated at room temperature for 3 minutes, and then centrifuged at 12,000 x g for 15 minutes at 4°C. The clear supernatant was transferred to a new Eppendorf tube and 500 μL of molecular grade isopropyl alcohol was added to precipitate the dissolved RNA, vortexed gently, and then incubated at room temperature for 10 minutes before centrifugation at 12,000 x g for 10 minutes at 4°C. The supernatant was discarded and the RNA pellet was washed with 1 mL of 75% molecular grade ethanol in diethylpyrocarbonate (DEPC) treated water twice to remove phenol-contaminants. The samples were then centrifuged at 7500 x g for 5 min at 4°C with the supernatant removed both times and after the second wash, the
samples were allowed to air dry briefly under a hood. RNA was then dissolved in DEPC water and incubated at 60°C for 10 minutes. To remove DNA contamination, a Turbo DNA-free Kit (Ambion, Foster City, CA) was utilized. A 10X Turbo DNase free digestion buffer and Turbo DNase I was added to the dilute RNA and incubated for 30 minutes at 37°C. DNase Inactivation Reagent was added, gently vortexed, allowed briefly to sit at room temperature, and centrifuged for 2 minutes at 10,000 x g. The supernatant was transferred to a new eppendorf tube and stored at -80°C until cDNA synthesis.

In order to assure RNA purity and sufficient removal of DNA or phenol contamination based on absorbance readings, RNA concentrations were assessed with a NanoDrop. 2.5 μg of RNA was then added to 0.2 mL Eppendorf tubes with 5X iScript reaction mix, Oligo (dt)20 primer, iScript reverse transcriptase (Bio-Rad, Hercules, CA), and DEPC water for reverse transcription to create single stranded DNA or cDNA. Samples were then incubated using the following temperature cycle: 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. cDNA was then quantified using a NanoDrop and stored at -20°C after synthesis. For Real-time Polymerase Chain Reaction (qRT-PCR), 250 ng of cDNA was added in triplicate to a 96 well plate with 10 pmol of forward and reverse primers of a specific gene of interest (Table 3.1), IQ SYBR Green Supermix (Bio-Rad, Hercules, CA), and DEPC water. The following temperature cycle protocol was then used to amplify the target gene (MyIQ, Bio-Rad, Hercules, CA): 3 minutes at 95° C, 40 cycles for 15 seconds at 95° C, 30 seconds at 60° C, 30 seconds at 72° C, 1 minute at 95° C, and 1 minute at 55° C, and 80 cycles for 15 s at 55° C. Analysis of the gene transcripts was done through the Comparative C_{T} method utilizing the 18s gene as the loading control gene (158).
Statistical Analysis

Analysis of the means for each group was conducted by a two-way ANOVA test using SigmaStat software (SPSS, Chicago, IL). A significance level of five percent was established and when differences of means were found to be significant (p < 0.05), the mean values were then compared using Fisher’s least significant difference (LSD) calculation.

Results

*Weight reduction reflects exercise training in both folate-deficient and control mice.* Weight gains in sedentary mice were significantly greater compared to the wheel exercised mice and the folate-deficient treadmill exercised but not the control treadmill exercised (p < 0.05). Initial weights of all mice were not significantly different from one another and there wasn’t an influence of diet on weight change (Figure 3.1).

*Wheel exercised mice ran more total miles compared to treadmill exercised mice.* The total distance in miles wheel exercised mice achieved during the training period was greater than treadmill exercised mice (p < 0.001). Total distance run by both folate-deficient wheel exercised and control diet wheel exercised mice were not statistically different from one another. Moreover, total distance for treadmill exercised mice did not differ between diet treatments. Average daily distance also did not significantly vary between wheel exercised groups (data not shown) (Figure 3.2).

*Hyperhomocysteinemia was attenuated by exercise in the folate-deficient group.* A folate deficient diet increased homocysteine concentrations >2 fold when compared to the sedentary control group (p < 0.001). Both treadmill and wheel exercise in the folate-deficient
group prevented an increase in homocysteine concentrations exhibited by the folate-deficient sedentary group (p < 0.001). When compared to their respective sedentary group, the treadmill and wheel exercised animals in the control diet in addition to the wheel exercised mice in the folate-deficient diet had reduced homocysteine concentrations when compared to the control diet sedentary group (p < 0.05) (Figure 3.3).

*Glutathione concentrations were not influenced by exercise or diet.* Plasma glutathione concentrations did not differ among the treadmill exercised, wheel exercised, and sedentary mice for both folate-deficient and control diets. Moreover, when dietary treatments were considered independent of exercise treatment, glutathione concentrations did not differ with the removal of folate. Also, the addition of an exercise treatment independent of diet did not significantly influence plasma glutathione concentrations (Figure 3.4).

*Hepatic GNMT activity was induced by exercise treatment in both dietary groups.* GNMT activity in the liver for the control wheel and folate-deficient treadmill groups increased by 37% and 23%, respectively, when compared to their sedentary counterparts (p < 0.05). When analyzed alone, the control treadmill (p < 0.05) and wheel exercised mice (p < 0.001) exhibited 21% and 37% increased GNMT activity, respectively, compared to the sedentary control group. Additionally, the folate-deficient wheel exercised mice displayed 23% greater GNMT activity when compared to the sedentary control diet group (p < 0.05). Moreover, both exercise treatment groups had greater GNMT activity compared to sedentary mice when dietary factors were not considered (p < 0.05; data not shown) (Figure 3.5).

*Liver BHMT activity was elevated in the folate-deficient diet group regardless of exercise status.* Hepatic BHMT activity increased about 2-fold across all groups in the folate-
deficient treatment when compared to the sedentary control (p < 0.05). Independent of exercise training, BHMT activity was also 70% greater in the folate-deficient group collectively when compared to the control group (p < 0.001; data not shown) (Figure 3.6).

*Free-access wheel running increased renal BHMT activity in both diet groups.* Renal BHMT activity was 2- and 2.3-fold higher in both control (p < 0.05) and folate deficient (p < 0.01) wheel exercised mice, respectively, when compared to the sedentary control. Additionally, wheel exercised mice had significantly increased kidney BHMT activity compared to treadmill exercised when diet was not factored (p < 0.01; data not shown) (Figure 3.7).

*Reduced liver MS activity was measured in both wheel exercised groups.* Hepatic MS activity was 1.6- and 2.1-fold lower in control (p < 0.05) and folate-deficient (p < 0.001) wheel exercised groups, respectively, when compared to the sedentary control diet. Folate-deficient treadmill mice also displayed 50% less MS activity compared to the sedentary control, however, the control treadmill group did not display similar activity (p < 0.05). Collectively, both the wheel (p < 0.001) and treadmill (p < 0.05) exercised groups displayed 1.7- and 1.3-fold reduction in MS activity when compared to the sedentary group independent of diet (data not shown) (Figure 3.8).

*Exercise did not influence hepatic mRNA expression in enzymes of interest.* Using the comparative C_{iT} method to quantify liver mRNA abundance, only the induction of GNMT in the control treadmill group displayed a 1.7 fold increase in mRNA expression (p < 0.05). Changes in gene expression of select methyl group metabolism genes were not significantly different from the control sedentary group (Table 3.2).
Discussion

The benefits of regular physical activity for human health have been well established in the literature particularly as a means to improve cardiovascular function; however, specific beneficial mechanisms are still inconclusive (12, 13, 159). Moreover, hyperhomocysteinemia has been previously suggested to be an independent risk factor for cardiovascular disease in a number of studies but it is unclear if elevated homocysteine concentrations are a direct cause for the disease (160-163). To our knowledge, this is the first study displaying reduced plasma homocysteine concentrations in a folate-deficient model previously shown to display hyperhomocysteinemia (10, 11). The duration and distance variation between the treadmill and wheel exercised groups did not influence changes in homocysteine concentrations suggesting that exercise may not be duration- or intensity-dependent; however, some of our other measures that influence homocysteine balance do appear to be duration- and intensity-dependent. Similar results have been observed in human populations where homocysteine concentrations were reduced by a variety of exercise modes (5, 7, 8, 140).

Mechanistically, enzymatic activity involved with the remethylation of homocysteine in the present study does not entirely reflect the observed reduction in homocysteine concentrations. A decrease in hepatic MS activity following exercise was evident in both control and folate-deficient mice. Based on reduced MS activity, homocysteine concentrations should theoretically increase due to decreased remethylation potential. In a heterozygous MS deficient mouse, Swanson and colleagues (2001) found that a disruption of MS activity lead to an increase in homocysteine concentrations, which conflicts with our observations of decreased homocysteine concentrations and reduced MS activity (164).
Moreover, Allen and others (1993) found that individuals with a folate-deficiency displayed increased dimethylglycine (DMG) and decreased betaine concentrations suggesting an increase in hepatic BHMT activity, which coincides with our hepatic BHMT findings in the folate-deficient diet group (165). Ohuchi and others (2009) also observed a dietary mediated increase in hepatic BHMT activity in addition to reduced homocysteine concentrations in rats (166). Thus, our data suggests that increased hepatic BHMT activity contributed to reduced homocysteine concentrations in folate-deficient exercised mice. Although hepatic BHMT and MS activity were elevated in folate-deficient sedentary animals, homocysteine concentrations remained elevated when compared to folate-deficient exercised animals.

Homocysteine is a product of all mammalian cells, however, remethylation and transsulfuration mechanisms to reduce its concentrations have been shown to be tissue specific. For instance, MS activity has been detected throughout most mammalian tissue whereas CBS activity occurs primarily in rat and human liver but has also been found in rat kidney tissue (167, 168). Moreover, BHMT expression is less distributed in mammalian tissue only being detected in rat and human liver and kidney tissue with specific activity in rat kidney being significantly less than humans (169, 170). In the present study, renal BHMT activity increased in the wheel exercised group suggesting a potential mode or duration effect on kidney homocysteine metabolism. With greater BHMT activity prevalent in humans compared to rodents, the influence of exercise may be more profound in humans and play a more significant role in reducing homocysteine concentrations.

As stated previously, the role of GNMT in the cell is to prevent aberrant methylation by transferring excess methyl groups to glycine and based on our observations, it may be
upregulated with treadmill or wheel exercise (39). Previous studies conducted in our lab have also shown that with increased GNMT activity, homocysteine concentrations are significantly reduced (56, 171, 172). A potential explanation for the increase in GNMT activity may be due to reduced 5-CH₃-THF concentrations, which has been previously shown to allosterically inhibit GNMT activity (173). The substrate 5-CH₃-THF comes from the irreversible reduction of 5,10-methylenetetrahydrofolate (5,10-CH₂-THF) via the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) (174). 5,10-CH₂-THF can also be used as a substrate for thymidylate synthase (TS), the enzyme responsible for the formation of thymidylate for DNA synthesis (175). Previous work has determined that in exercised mice and rats, the concentration of mitochondria and mitochondrial DNA increased in skeletal muscle, potentially increasing TS activity for DNA synthesis (176, 177). Therefore, with increased need of thymidylate for DNA synthesis during exercise, TS may compete with MTHFR reducing 5,10-CH₂-THF availability for folate-dependent remethylation and therefore reduce the allosteric inhibition of GNMT (178).

Remethylation patterns in the liver, the primary site for amino acid metabolism, did not coincide with the reduction in homocysteine concentrations observed in exercised mice for this study (179). Other mechanisms to reduce homocysteine may have been more prominent with exercise treatment, such as decreased methionine substrate availability for transmethylation. Dohm and colleagues (1981) observed an increase in methionine concentrations in rat plasma, muscle, and liver immediately following treadmill running or forced swimming and proposed this increase was due to elevated muscle catabolism for energy expenditure (152). Others have also shown similar increases in plasma methionine
concentrations during cycle ergometer training but also displayed a significant reduction in the hours post-exercise suggesting in increase in protein synthesis and thus limiting the availability of methionine for transmethylation (150, 151, 153). Reductions in methionine availability in the bloodstream during various modes of exercise may have important implications for dietary methionine requirements to support both muscle and methyl group metabolism.

The irreversible transsulfuration of homocysteine provides cysteine for a number of products, such as glutathione, and α-ketobutyrate which can be metabolized to CO₂ (42, 77). As an antioxidant in the body, glutathione has been shown to exist primarily in its oxidized form during exercise as a response to increased oxidative stress (180). Moreover, Sastre and others found similar oxidation effects of glutathione during exercise but 1 hour after exercise, resting levels of reduced glutathione were measured and total concentrations of glutathione were unchanged (181). Although initial total glutathione was not analyzed in the present study, similar results were observed as there was no difference in glutathione concentrations between the sedentary and exercised groups. As glutathione is not the only fate of cysteine through the transsulfuration pathway, this measurement cannot solely refute that catabolism of homocysteine is not perturbed with the addition of exercise. Future studies need to assess CBS abundance and activity in order to establish the influence of exercise on the transsulfuration of homocysteine.

From the present study, it was evident that exercise normalized homocysteine concentrations in a mouse model known to experience hyperhomocysteinemia. Although liver enzymatic remethylation mechanisms did not completely reflect the observed changes
in homocysteine, renal enzymatic activity corresponded to changes in homocysteine concentrations potentially establishing an important role of kidney homocysteine metabolism during exercise. Future research will need to establish the connection between the transsulfuration pathway and exercise in regulating homocysteine metabolism in addition to the activity changes of pertinent transmethylation enzymes, such as PEMT and GAMT, to understand how normal cellular methylation patterns might be perturbed with exercise. Additionally, understanding the influence of frequency, mode, duration, and intensity on homocysteine concentrations may help to determine a potential dose response. Furthermore, understanding the impact exercise has on populations with known perturbations in methyl group and homocysteine metabolism, such as diabetics, vegans, and individuals with genetic polymorphisms, may serve as an aid in normalizing these mechanisms.
Table 3.1: Forward and reverse primer sequences for folate, methyl-group, and homocysteine metabolism enzymes (Real-time PCR; Integrated DNA Technologies).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Primers Sequences</th>
<th>Reverse Primers Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNMT</td>
<td>TTG AAG AAG CCA ACT GGT TGA CGC</td>
<td>TGC AAT GTT CTT TAG TGC CAG CCG</td>
</tr>
<tr>
<td>MS</td>
<td>ATG GCA CAG GAG GGA AGA AAG TCA</td>
<td>TGC CCT TCA CAA GAG CAT ACT CCA</td>
</tr>
<tr>
<td>BHMT</td>
<td>AGT TCG TCA GCT TCA TCG GGA GTT</td>
<td>AAG CAG CTT CGT TGA CTT TCT GCC</td>
</tr>
<tr>
<td>CBS</td>
<td>TCA CAT TCT GGA CCA GTA CCG CAA</td>
<td>ACT TCT CCT TCA GCT TTC TGG CGA</td>
</tr>
<tr>
<td>PEMT</td>
<td>TAT GAT GAG CCA GCC CAA GAT GGA</td>
<td>GAA GCT GGA CAG CAC AAA CAC GAA</td>
</tr>
<tr>
<td>18s (Ribosomal RNA)</td>
<td>TCA TGC AGA ACC CAC GAC AGT ACA</td>
<td>TGT TGT CTA GAC CGT TGG CCA GAA</td>
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</table>
Table 3.2: Hepatic mRNA mean fold induction relative to sedentary control diet group. Data are represented as means ± S.E. (n = 4-6). Values followed by * are statistically different from the sedentary control diet group (p < 0.05).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sedentary Treadmill</th>
<th>Sedentary Wheel</th>
<th>Folate Deficient Diet Treadmill</th>
<th>Folate Deficient Diet Wheel</th>
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<tbody>
<tr>
<td></td>
<td>Fold</td>
<td>p-value</td>
<td>Fold</td>
<td>p-value</td>
</tr>
<tr>
<td>GNMT</td>
<td>1.00 ± 0.22</td>
<td></td>
<td>1.26 ± 0.27 0.473</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.71 ± 0.22 0.049*</td>
<td></td>
</tr>
<tr>
<td>BHMT</td>
<td>1.00 ± 0.38</td>
<td></td>
<td>1.06 ± 0.35 0.913</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.42 ± 0.32 0.422</td>
<td></td>
</tr>
<tr>
<td>CBS</td>
<td>1.00 ± 0.30</td>
<td></td>
<td>1.07 ± 0.31 0.878</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.06 ± 0.31 0.903</td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>1.00 ± 0.31</td>
<td></td>
<td>1.53 ± 0.79 0.514</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.16 ± 0.38 0.756</td>
<td></td>
</tr>
<tr>
<td>PEMT</td>
<td>1.00 ± 0.10</td>
<td></td>
<td>1.13 ± 0.22 0.590</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.98 ± 0.12 0.923</td>
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Figure 3.1: Initial weight of mice prior to exercise treatment and final weight of mice before euthanasia for control and folate-deficient diet sedentary, treadmill exercised, and wheel exercised treatment groups. Data are expressed as means ± S.E (n= 4-6). Bars denoted with different letters are statistically different within the group (p < 0.05).
Figure 3.2: Total distance run by control and folate-deficient diet treadmill and wheel exercised mice. Data are expressed at means ± S.E. (n = 5).
Figure 3.3: Homocysteine concentrations for control and folate-deficient diet sedentary, treadmill exercised, and wheel exercised mice. Data are expressed as means ± S.E. (n = 4-6). Bars labeled with different letters are significantly different (p < 0.001). Bars labeled with an * are statistically lower than the control diet sedentary group (p < 0.05).
Figure 3.4: Glutathione concentrations for control and folate-deficient diet sedentary, treadmill exercised, and wheel exercised mice. Data are represented as means ± S.E. (n = 4-6).
Figure 3.5: Glycine \( N \)-methyltransferase (GNMT) activity for control and folate-deficient diet sedentary, treadmill exercised, and wheel exercised mice. Data are expressed as means ± S.E. (n = 4-6). Bars not followed by the same letter are statistically different (p < 0.05). Bars represented by an * are significantly greater than the sedentary control diet group (p < 0.05).
Figure 3.6: Hepatic betaine-homocysteine S-methyltransferase (BHMT) activity for control and folate-deficient diet sedentary, treadmill exercised, and wheel exercised mice. Data are represented as means ± S.E. (n = 4-6). Bars not denoted by the same letter are significantly different (p < 0.05).
Figure 3.7: Renal betaine-homocysteine $S$-methyltransferase (BHMT) activity for control and folate-deficient diet sedentary, treadmill exercised, and wheel exercised mice. Data are represented as means ± S.E. ($n = 4$-$6$). Bars not labeled with the same letters are statistically different ($p < 0.05$).
Figure 3.8: Methionine Synthase (MS) activity for control and folate-deficient diet sedentary, treadmill exercised, and wheel exercised mice. Data are represented as means ± S.E. (n = 3-6). Bars not represented by the same letter are statistically different (p < 0.05).
CHAPTER 4 – GENERAL CONCLUSIONS

Proper methyl group and homocysteine metabolism is imperative for human health as evident by the number of pathologies associated with disturbances in these pathways including diabetes, cancer, and cardiovascular disease (40, 143, 182). A number of mechanisms have been put forth to modulate perturbed methyl group and homocysteine metabolism such as B-vitamin supplementation to enhance homocysteine reduction and the use of antifolates in limiting cancer progression (116, 183). Although fairly controversial in the human population, the present study demonstrated the potential benefits of exercise by reducing and normalizing homocysteine concentrations in a folate-sufficient and -deficient mouse model, respectively. Utilizing this model helped to limit high variability seen in a number of human studies including uncontrolled dietary intake, differences in subject training status, and mode of exercise tests (123, 184, 185). Moreover, this model allowed us to collect hepatic and renal tissue, a significant limitation in the human population for a thorough assessment of homocysteine and methyl group metabolism.

It is well established that exercise promotes cardiovascular health as demonstrated in both human and rodent models but the protective mechanisms are still fairly controversial (12, 13, 159, 186). Moreover, the elevation in homocysteine concentrations has been implicated as an independent risk factor for cardiovascular disease although the current mechanism regarding the influence of homocysteine on the cardiovascular system is still unknown (160-163). Taken together, the homocysteine lowering effects shown in this study for both a folate-deficient and -sufficient mouse model may potentially explain one protective mechanism of exercise in cardiovascular health. Future research will need to
examine vascular integrity and function in order to establish a direct correlation between the two.

Consistent with previous research in our lab and others, the present study clearly demonstrated the significant influence a dietary limitation such as folate restriction has on homocysteine concentrations (10, 11, 40). As a folate deficiency with concomitant hyperhomocysteinemia has been specifically linked to disorders such as coronary heart disease and Alzheimer’s, exercise may play an important role in reducing the incidence of these pathologies (187, 188). Although homocysteine concentrations in folate-deficient mice were normalized with exercise, other cellular processes, like de novo thymidylate synthesis, absolutely require folate (19). Therefore, exercise should only be viewed as a potential modulator of homocysteine concentrations typically seen in folate deficiencies and not as a dietary replacement. Moreover, this model serves as a platform for other populations prone to hyperhomocysteinemia independent of folate such as individuals with genetic polymorphisms and vegans.

The enzymatic changes observed with exercise in both folate-deficient and -sufficient models did not offer major insight into the reduction of homocysteine concentrations as the sedentary groups displayed similar or elevated remethylation patterns, as seen with hepatic betaine homocysteine S-methyltransferase (BHMT) and methionine synthase (MS) activity, respectively. However, renal enzymatic function may become more important for reducing homocysteine concentrations with exercise due to increased BHMT activity observed in wheel exercised mice. Moreover, a decrease in methionine availability is one proposed mechanism in the reduction of homocysteine with exercise. It has previously been shown that
amino acid availability fluctuates during and after exercise due to changes in energy requirements and protein metabolism (150-153). Specifically, the enhanced protein synthesis seen post exercise would then warrant the observed decrease in plasma methionine. Future studies will need to assess changes in methionine concentrations to see the extent of incorporation into proteins during exercise in addition to its availability for transmethylation reactions within cells. Moreover, previous work has demonstrated a significant increase in homocysteine concentrations with methionine loading (189). Thus, examining the influence exercise has on homocysteine concentrations with methionine loading may offer some insight into methionine use during exercise.

Another aspect of methyl group and homocysteine metabolism that requires more investigation is the transsulfuration pathway. Although changes in glutathione, a major product of transsulfuration, were not different among the sedentary and exercise treatments, enzymatic function in addition to other metabolites of transsulfuration may have been influenced by exercise. For instance, cysteine, the precursor for glutathione production, has previously been shown to decrease significantly in exercised rat plasma indicating a potential increase in the oxidation of cysteine for energy or utilization in protein synthesis (190). Thus, cystathionine beta-synthase (CBS) and cystathionine γ-lyase (CGL) abundance and activity should be measured to understand the role of transsulfuration in homocysteine metabolism during exercise.

Although this study further emphasizes the importance of exercise in disease prevention, it also provides potential insight into homocysteine management in populations with diabetes, genetic polymorphisms, and diet restrictions that experience
hyperhomocysteinemia. Therefore, exercise may be an additional mean of combating pathologies associated with hyperhomocysteinemia in these populations where dietary or medical intervention is limited, such as in individuals with the methylenetetrahydrofolate reductase (MTHFR) polymorphism. Likewise, dietary choices of vegans limit essential B-vitamin consumption, specifically vitamin B$_{12}$, and exercise could potentially mediate the elevation in homocysteine concentrations experienced with perturbed homocysteine remethylation. However, exercise may also be important in controlling homocysteine concentrations in normal populations as a preventative mechanism in limiting the development of disease. Future research should be aimed at understanding the influence of exercise on methyl group and homocysteine metabolism in regards to mode, frequency, and intensity across all populations.
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