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Exercise prevents hyperhomocysteinemia in a dietary folate-restricted mouse model.

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Abstract
Hyperhomocysteinemia is a condition that results from altered methyl group metabolism and is associated with numerous pathological conditions. A number of nutritional and hormonal factors have been shown to influence circulating homocysteine concentrations; however, the impact of exercise on homocysteine and methyl group balance is not well understood. Our hypothesis was that exercise represents an effective means to prevent hyperhomocysteinemia in a folate-independent manner. The purpose of this study was to determine the influence of exercise on homocysteine metabolism in a dietary folate-restricted mouse model characterized by moderate hyperhomocysteinemia. Female outbred mice (12 weeks old) were assigned to either a sedentary or free-access wheel exercise group. Following a 4-week acclimation period, half of the mice in each group were provided a folate-restricted diet for 7-weeks prior to euthanasia and tissue collection. As expected, folate-restricted sedentary mice exhibited a 2-fold increase in plasma total homocysteine concentrations; however, exercise completely prevented the increase in circulating homocysteine concentrations. Moreover, exercise reduced plasma homocysteine concentrations 36% within the group fed only the control diet. The prevention of hyperhomocysteinemia by exercise appears, at least in part, to be the result of increased folate-independent homocysteine remethylation owing to a 2-fold increase in renal betaine homocysteine S-methyltransferase. To our knowledge, this is the first report demonstrating the prevention of hyperhomocysteinemia by exercise in a dietary folate-restriction model. Future research will be directed at determining if exercise can have a positive impact on other nutritional, hormonal, and genetic models of hyperhomocysteinemia relevant to humans.

Keywords
folate, hyperhomocysteinemia, exercise, betaine-homocysteine S-methyltransferase, mouse

Exercise prevents hyperhomocysteinemia in a dietary folate-restricted mouse model

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Abbreviations:

BMT; betaine-homocysteine S-methyltransferase

CBS; cystathionine β-synthase

GNMT; glycine N-methyltransferase

MS; methionine synthase

MTHFR; 5,10-methylene-tetrahydrofolate reductase

PEMT; phosphatidylethanolamine N-methyltransferase

SAH; S-adenosylhomocysteine

SAM; S-adenosylmethionine

THF; tetrahydrofolate

Abstract

Hyperhomocysteinemia is a condition that results from altered methyl group metabolism and is associated with numerous pathological conditions. A number of nutritional and hormonal factors have been shown to influence circulating homocysteine concentrations; however, the impact of exercise on homocysteine and methyl group balance is not well understood. Our hypothesis was that exercise represents an effective means to prevent hyperhomocysteinemia in a folate-independent manner. The purpose of this study was to determine the influence of exercise on homocysteine metabolism in a dietary folate-restricted mouse model characterized by moderate hyperhomocysteinemia. Female outbred mice (12 wk old) were assigned to either a sedentary or free-access wheel exercise group. Following a 4-wk acclimation period, half of the mice in each group were provided a folate-restricted diet for 7-wk prior to euthanasia and tissue collection. As expected, folate-restricted sedentary mice exhibited a 2-fold increase in plasma total homocysteine concentrations; however, exercise completely prevented the increase in circulating homocysteine concentrations. Moreover, exercise reduced plasma homocysteine concentrations 36% within the group fed only the control diet. The prevention of hyperhomocysteinemia by exercise appears, at least in part, to be the result of increased folate-independent homocysteine remethylation owing to a 2-fold increase in renal betaine homocysteine *S*-methyltransferase. To our knowledge, this is the first report demonstrating the prevention of hyperhomocysteinemia by exercise in a dietary folate-restriction model. Future research will be directed at determining if exercise can have a positive impact on other nutritional, hormonal, and genetic models of hyperhomocysteinemia relevant to humans.

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1. Introduction

The maintenance of the folate-dependent one-carbon pool and methyl group metabolism is essential for optimization of health. Perturbations of these interrelated metabolic pathways have been implicated in a number of diseases, including cancer development, cardiovascular disease, neural tube defects, and cognitive disorders [1-4]. Homocysteine is an important intermediate in methyl group metabolism and is partially dependent on folate/ B_{12} for its metabolism. Hyperhomocysteinemia, a condition that can result from a lack of methyl group donors, cofactors, and/ or relevant genetic anomalies, has been shown to be an independent risk factor in the development of cardiovascular disease [5].

Homocysteine is a product of transmethylation reactions involving *S*-adenosylmethionine (SAM), the activated form of methionine, in which a methyl group is donated to a number of acceptors, including proteins, lipids, and nucleic acids (Fig. 1) [6]. Homocysteine can be remethylated back to methionine by folate-dependent or -independent mechanisms, or undergo irreversible catabolism by transsulfuration. Folate-dependent remethylation utilizes folic acid in its most reduced form to transfer a methyl group to homocysteine and generate methionine via the vitamin B_{12}-dependent enzyme methionine synthase (MS). Conversely, folate-independent remethylation of homocysteine utilizes the enzyme betaine-homocysteine *S*-methyltransferase (BHMT) and a methyl group from betaine, a compound derived from the oxidation of choline. Transsulfuration of homocysteine by the vitamin B_6-dependent enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase leads to irreversible catabolism and the eventual formation of cysteine. Thus, homocysteine balance and the prevention of

hyperhomocysteinemia are dependent on a number of substrates, cofactors, and the proper expression and function of key enzymes.

As the regulation of homocysteine balance is vital to maintain optimal health, the establishment of homocysteine management-based therapies is necessary to prevent or treat diseases related to hyperhomocysteinemia. Recent studies examining the role of exercise as a potential means to reduce circulating homocysteine concentrations have been inconclusive, owing in large part to the variations in study design and exercise regimes [7-15]. Moreover, discrepancies within these human studies, including B-vitamin and subject training status, as well as variations in mode, intensity, and duration of test exercises, limit the strength of their conclusions [16, 17]. Mechanistically, reductions in homocysteine concentrations by exercise may be related to increased protein turnover owing to increased plasma methionine concentrations during exercise, followed by reduced concentrations below basal levels after exercise [18-21]. This fluctuation in methionine availability for methyl group metabolism may be due, in part, to the increased need of methionine for muscle anabolism, potentially resulting in diminished homocysteine production [17, 21]. However, exercise also increases the demand of vitamin B_6 to support increased muscle catabolism, thereby potentially limiting its availability for transsulfuration and subsequently resulting in homocysteine accumulation [22].

Our hypothesis was that exercise represents an effective means to prevent hyperhomocysteinemia in a folate-independent manner. This was based on our previous research demonstrating that a gluconeogenic state and related hormonal alterations, similar to what is exhibited as a function of exercise, results in reduced homocysteine concentrations via

enhanced folate-independent remethylation of homocysteine, as well as increased catabolism [23-26]. The aim of the present study was to assess the influence of voluntary exercise on homocysteine balance using a folate-restricted mouse model of hyperhomocysteinemia. This moderate hyperhomocysteinemia model was utilized to represent populations that experience poor folate absorption or intake, as well as individuals with relevant polymorphisms associated with modestly high circulating homocysteine concentrations, such as the 5,10-methylenetetrahydrofolate reductase (MTHFR) C677T gene [27].

2. Methods and materials

2.1. Chemicals and reagents.

Reagents were obtained as follows: [$^{14}CH_3$]-betaine, Moravek; DL-homocysteine thiolactone, Sigma-Aldrich Chemical; 5-[$^{14}CH_3$]-tetrahydrofolate, Amersham Pharmacia; S-adenosyl-L-[*methyl*-^{3}H] methionine, New England Nuclear. All other reagents were of analytical grade.

2.2. Animals and diets.

All animal procedures and protocols were approved by and conducted in accordance with guidelines established by Iowa State University Laboratory Animal Resources. Female intercrossing outbred mice (9-10 wk of age) were obtained from Harlan (Indianapolis, IN) and initially housed in groups of 2 or 3 in a 12-h light: dark cycle and provided an AIN-93G semi-purified diet (Table 1) and water ad libitum [28]. No antibiotics were added to the diets or drinking water, resulting in a moderate degree of folate deficiency as we have previously reported [24]. After an acclimation period of 3 d, mice were randomly assigned to one of 2 groups: sedentary or free-access wheel exercised. Wheel exercised-mice were housed individually to obtain accurate distance calculation. For the duration of the study, wheel exercised-mice had free-access to their wheels 24 h/ d for 5 d/ wk. After 4 wk, half of the mice in each group were switched to a folate-restricted diet resulting in 4 groups: sedentary with control diet; sedentary with folate-restricted diet; wheel-exercised with control diet; and wheel-exercised with folate-restricted diet. After 11 wk, mice were fasted for 12 h and given an

intraperitoneal injection of freshly prepared ketamine: xylazine (90: 10 mg/kg body wt). Euthanasia consisted of exsanguination and removal of major organs for their subsequent processing as described previously [23-26]. Heparinized whole blood was collected via cardiac puncture, centrifuged at 4,000 *g* for 6 min, and plasma was stored at -20°C for subsequent homocysteine analysis. Liver tissue was rapidly removed and 0.5 g portions were homogenized in 2 ml of an ice-cold buffer containing 10 mM sodium phosphate (pH 7.0), 1 mM EDTA, 1 mM sodium azide, 0.25 M sucrose, and 0.1 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 20,000 *g* for 30 min at 4°C and the resulting supernatant was stored at -80°C for enzyme activity analysis. One kidney was removed, homogenized in 4 vol of the same buffer, and extracts were stored similar to liver samples. Total soluble protein concentrations were determined utilizing the Pierce Bicinchoninic Acid method (Thermo Scientific) with bovine serum albumin as the standard.

2.2. Determination of homocysteine concentrations.

Plasma homocysteine concentrations were determined as described by Ubbink et al. [29] with modifications [25]. For intracellular homocysteine determination, hepatic and renal tissue were homogenized in 2 volumes of 0.4 M perchloric acid, centrifuged at 9,000 *g* for 10 min at 25°C, and the resulting supernatant neutralized with 8 M potassium hydroxide and treated in the same manner as the plasma samples [30]. Both intracellular and plasma samples were incubated at 4°C for 30 min in a solution containing 1 mM N-acetylcysteine as an internal standard and 10% tributylphosphine in dimethylformamide. Addition of 10% trichloroacetic acid with 1 mM EDTA was used to stop the reaction and centrifuged at 1,000 *g* for 5 min at 4°C. For

derivatization, the supernatant was collected and added to a solution containing 0.125 M borate buffer (pH 9.5), 0.1% 4-fluoro-7-sulfobenzofurazan, and 1.55 M sodium hydroxide. Homocysteine detection and quantification was performed by HPLC in combination with fluorescence detection using a μBondapak C_{18} Radial-Pak column (Waters Associates) and a mobile phase containing 4% acetonitrile in 0.1 M potassium phosphate buffer (pH 2.1).

2.3. Enzyme activity determinations.

Measurement of BHMT activity was based on the method described by Garrow [31] and performed in triplicate. Protein aliquots of 40 and 100 μg for hepatic and renal tissue, respectively, were added to a reaction mixture containing the following: 50 mM [$^{14}CH_3$]-betaine, 100 mM DL-homocysteine thiolactone, 500 mM Tris (pH 7.5), 50 g/L bovine serum albumin, 10% 2-mercaptoethanol solution, and deionized water. Following incubation at 37°C for 1 h, the reaction was terminated with 2.5 ml of deionized water and samples were immediately applied to Dowex 1×4 (OH form) resin columns. Eluted fractions were collected in scintillation vials and radioactivity measured by liquid scintillation counting.

MS activity measurements were performed as described [32] with 600 μg protein added to 100 μl of a reaction mixture containing freshly prepared 100 mM DL-homocysteine thiolactone, 1.3 mM cyanocobalamin, 500 mM sodium phosphate buffer (pH 7.5), 10 mM S-adenosylmethionine, 82.4 mM 2-mercaptoethanol, 1 mM dithiothreitol, 15 mM 5-[$^{14}CH_3$]-tetrahydrofolate, and deionized water. Following incubation at 37°C for 1 h, the reaction was terminated with 800 μl

of ice-cold deionized water, applied to AG 1-X8 resin (Cl form) column, and the effluent (3 ml total) was collected in vials for liquid scintillation counting.

2.4. Statistical analyses.

Means for individual treatment groups were analyzed by two-way ANOVA using SigmaStat software (SPSS, Chicago, IL). A Student's *t*-test was used to compare sedentary and exercise means within a diet group. When means were statistically different ($P \leq 0.05$), Fisher's least significant difference procedure was used for comparison [33].

3. Results

3.1. Exercise decreased weight gain in both control-fed and folate-restricted mice.

Initial body weight measurements of mice across all groups were not statistically different. However, control diet exercised mice and folate-restricted diet exercised mice exhibited 24 and 18% decrease, respectively, in final body weight (Table 2). Folate-restriction was without effect on weight gain in either the sedentary or exercised group. Thus, this experimental design can be considered a moderate degree of folate deficiency, similar to our previous report [23]. The total distance (km) of exercise was not significantly different between control diet and folate-restricted diet groups.

3.2. Exercise prevented hyperhomocysteinemia in the folate-restricted dietary treatment group.

As expected, a folate-restricted diet increased plasma homocysteine concentrations >2-fold in the sedentary group (Fig. 2). However, the addition of wheel exercise in the folate-restricted diet completely prevented the increase in homocysteine concentrations compared to the folate-restricted diet sedentary group. Moreover, exercise alone decreased plasma homocysteine concentrations 36% in the control diet group.

3.3. Folate-restriction and exercise modulated hepatic homocysteine remethylation enzymes and intracellular homocysteine concentrations.

A folate restricted diet increased the activity of BHMT, but was without effect on MS activity in the liver (Table 3). In contrast, exercise reduced MS activity in both diet groups, but was without effect on hepatic BHMT activity. Hepatic intracellular homocysteine concentrations were not statistically different when all four mean values were compared; however, the exercised groups taken together exhibited diminished homocysteine concentrations compared to the sedentary groups ($P = 0.02$).

3.4. Renal BHMT activity was increased by exercise.

Although the amount of BHMT activity in renal tissue is significantly lower than the liver, it was markedly influenced by exercise (Fig. 3). Exercise increased renal BHMT activity in the control and folate-restricted groups, 101 and 60%, respectively. In contrast to the liver, renal BHMT was not altered by a folate-restricted diet alone.

4. Discussion

The benefits of exercise for human health have been well documented, particularly with respect to improving cardiovascular function [34]. Because hyperhomocysteinemia has been shown to be an independent risk factor for cardiovascular disease [5], identifying and understanding intervention strategies to promote homocysteine balance is an important goal for disease management. To our knowledge, this is the first report clearly demonstrating that exercise can completely prevent an increase in circulating homocysteine concentrations in a dietary folate-restricted mouse model of hyperhomocysteinemia, thereby supporting our original hypothesis.

Although hyperhomocysteinemia has been shown to be an independent risk factor for cardiovascular disease, it is unclear what influence elevated homocysteine concentrations have on vasculature and disease progression [35]. There is little doubt that hyperhomocysteinemia plays a role in the development of cardiovascular disease. This is not only supported by human population studies identifying it as an independent risk factor, but strong evidence resides in animal models with diet- and/ or genetic-based elevations in homocysteine concentrations [36, 37]. However, clinical trials targeting homocysteine management by the utilization of B-vitamin supplementation as a means to lower circulating homocysteine concentrations have not been as effective as anticipated [38-41]. Numerous reviews have debated the various explanations for these findings and the associative vs. causal role of homocysteine in vascular disease [42-44]. Nonetheless, it is clear that well-define indices of vascular disease result from animal studies utilizing genetic- and dietary-induced elevations in the concentration of plasma homocysteine .

The specific mechanism by which exercise prevents hyperhomocysteinemia owing to a folate-restricted diet is not completely clear. Homocysteine balance depends on its production from SAM-dependent transmethylation reactions, remethylation by folate-dependent and folate-independent pathways, and irreversible catabolism through the transsulfuration pathway. Here, we evaluated many of these possibilities by determining the expression and function of key regulatory enzymes involved in homocysteine production, remethylation, and catabolism. This analysis did not provide any additional mechanistic insight with respect to the positive effect of exercise on preventing hyperhomocysteinemia. The increase (53%) in mean hepatic BHMT activity by exercise in the control diet group did not reach statistical significance ($P = 0.13$), whereas MS activity was reduced in both groups by exercise. Interestingly, a folate-restricted diet alone resulted in significant 111% elevations in hepatic BHMT activity. Our previous folate-restriction studies using a rat model did not exhibit elevations in hepatic BHMT activity to the extent demonstrated with this mouse model [23]. Others have reported that dietary-mediated alterations in hepatic BHMT activity resulted in decreased homocysteine concentrations [45]. Moreover, it has been reported that folate-deficiency results in increased concentrations of dimethylglycine and decreased circulating concentrations of betaine, indicating a potential elevation in hepatic BHMT activity [46].

A significant amount of homocysteine metabolism occurs in the kidney [47] and this tissue has been shown to be a major factor under other conditions, such as diabetes, that are characterized by aberrant homocysteine balance [30]. Although the expression of BHMT in the rodent kidney is quite low[48], we found that exercise resulted in a significant increase in renal BHMT activity in the control diet group, as well as the folate-restricted group. It is not clear whether these

alterations in renal BHMT activity are biologically sufficient to explain the prevention of hyperhomocysteinemia by exercise.

Prolonged exercise is characterized by numerous changes in circulating hormones that ultimately promote gluconeogenesis and utilization of free fatty acids. This shift is also reflected in other gluconeogenic states, such as diabetes. We and others have demonstrated that a diabetic state or administration of synthetic glucocorticoid compounds has a major impact on methyl group and homocysteine metabolism [23-26, 49-52]. A consistent finding from these reports is a reduction in circulating homocysteine concentrations owing to an increase in folate-independent remethylation (i.e., BHMT) and catabolism of homocysteine through the transsulfuration pathway. This finding was the basis for our hypothesis and supports our results that exercise can prevent hyperhomocysteinemia that is the result of dietary folate restriction.

It also remains a possibility that the maintenance of homocysteine balance by exercise in folate-restricted mice may not be the result of direct changes in homocysteine metabolism, but rather alterations in methionine and/ or cysteine requirements as a function of protein metabolism and energy needs. Increased muscle anabolism following exercise may increase the methionine requirement for protein synthesis, thereby limiting its availability for SAM-dependent transmethylation reactions and subsequently decreasing homocysteine production. Alterations in intracellular methionine concentrations owing to exercise have been reported in both animal and human studies [19-22]. Transsulfuration of homocysteine provides cysteine and α-ketobutyrate, both of which can be utilized in energy production and may have increased importance in supplying the cell with energy during exercise [53]. Previous research found plasma cysteine

concentrations were decreased in exercised rats, indicating a potential increase in the utilization of cysteine for both protein synthesis and/or as a source of energy [54].

In summary, we have demonstrated that exercise represents an effective strategy to maintain homocysteine balance in a diet-mediated model of hyperhomocysteinemia. A limitation of this study and goal for future research is to determine the exercise dose (i.e., time, intensity) required to effectively prevent hyperhomocysteinemia, as well as potential adverse vascular outcomes. We have found in preliminary studies that mice subjected to a treadmill regime consisting of a specified intensity for a defined time period was nearly as effective as ad libitum wheel exercise, even though the total distance exercise was markedly less. Future research also needs to be directed at determining the precise signal and mechanism for the impact of exercise on prevention of hyperhomocysteinemia. Although additional research is required to define the precise relation between exercise and homocysteine balance, the impact of our observations has significant health implications for many individuals. We anticipate that our findings will stimulate future animal and human studies directed at evaluating the impact of exercise on other dietary, hormonal, and genetic models of hyperhomocysteinemia.

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Table 1 – Ingredient composition of the basal and folate-restricted diets fed to mice

Components[a]	g/kg diet
Casein, vitamin-free	100.0
Cornstarch	402.0
Glucose, monohydrate	393.0
Corn oil	50.0
Vitamin mix[b]	10.0
Mineral mix[c]	40.0
L-methionine	3.0
Choline bitartrate	2.0

[a] All diet ingredients were purchased from Harlan Teklad (Madison, WI), except L-methionine and choline bitartrate (Sigma Aldrich).

[b] AIN-93-VX formulation (Harlan Teklad). For folate-restricted mice, a customized vitamin mix devoid of folate was used (Harlan Teklad).

[c] AIN- 93G-MX formulation (Harlan Teklad).

Table 2 – Body weights and distance exercised from control and folate-restricted rats with and without exercise

	Control		Folate-restricted		2-Way ANOVA P-values		
	- Ex	+ Ex	- Ex	+ Ex	Diet	Ex	Diet × Ex
Initial Weight (g)	28.7±0.7	27.6±0.7	28.3±0.3	27.2±0.6	NS	NS	NS
Final Weight (g)	35.7±2.2[a]	27.0±1.1[b]	35.3±1.1[a]	29. ±1.0[b]	0.558	<0.001	0.384
Total Distance (km)	NA	930±101	NA	756±102			

Data are means ± S.E., n = 5-6. Means within a column without a common superscript letter differ, $P \leq 0.05$. Ex, exercise; NA, not applicable; NS, not significant.

Table 3 – Hepatic activity of betaine-homocysteine S-methyltransferase (BHMT) and methionine synthase (MS), and intracellular homocysteine (Hcy) concentrations from control and folate-restricted rats with and without exercise

	Control		Folate-restricted		2-Way ANOVA P-values		
	- Ex	+ Ex	- Ex	+ Ex	Diet	Ex	Diet × Ex
BHMT (pmol/min·mg)	87±15[a]	133±21[a,b]	184±22[b]	159±25[b]	0.013	0.640	0.125
MS (pmol/min·mg)	108±11[a]	67±12[b,c]	89±8[a,b]	51±8[c]	0.058	<0.001	0.884
Hcy (nmol/g)	6.5±0.6	4.9±0.4	5.7±0.4	5.0±0.5	0.438	0.019	0.317

Data are means ± S.E., n = 5-6. Means within a column without a common superscript letter differ, $P \leq 0.05$. Ex, exercise.

Figure Legends

Fig. 1 – Methyl group and homocysteine metabolism. Enzymes are shown in black boxes, whereas vitamin substrates and/or cofactors are shown in gray boxes. Abbreviations are: betaine-homocysteine *S*-methyltransferase [BHMT]; cystathionine β-synthase [CBS]; dimethylglycine [DMG]; methionine synthase [MS]; methyltransferases [MTs]; 5,10-methylene-THF reductase [MTHFR]; *S*-adenosylhomocysteine [SAH]; SAH hydrolase [SAHH]; *S*-adenosylmethionine [SAM]; tetrahydrofolate [THF]; and methyl acceptor [X]. In addition to THF, this series of interrelated pathways are dependent on a number of other B-vitamins, including riboflavin [B_2], vitamin B_6, and vitamin B_{12}.

Fig. 2 – Plasma homocysteine concentrations of control and folate-restricted diet sedentary and wheel-exercised mice. Half of the mice in each diet group were allowed access to an exercise wheel for 4 wk, after which they were then fed either a control diet or a diet without folate in the vitamin mix. After an additional 7 wk, plasma samples were obtained for the measurement of total homocysteine concentrations. Values are means ± SE; n = 5-6. Bars without a common letter differ, $P \leq 0.05$. Bars denoted with an asterisk [*] are different from control diet sedentary group, $P \leq 0.05$. Two-way ANOVA: diet, $P = 0.002$; exercise, $P < 0.001$; interaction, $P = 0.010$.

Fig. 3 - Renal betaine-homocysteine *S*-methyltransferase [BHMT] activity of control and folate-restricted diet sedentary and wheel-exercised mice. Kidney samples from the control and folate-restricted mice with or without exercise were homogenized for enzyme activity determination. Values are means ± SE; n = 5-6. Bars without a common letter differ, $P \leq 0.05$. BHMT activity

is defined as pmol/ [min • mg protein]. Two-way ANOVA: diet, $P = 0.246$; exercise, $P = 0.007$; interaction, $P = 0.822$.