



Acute exercise alters homocysteine plasma concentration in an intensity-dependent manner due increased methyl flux in liver of rats

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ABSTRACT

Purpose: We aimed to determine the effects of different intensities of acute exercise on Hcy plasma levels, and the exercise-induced changes in Hcy liver metabolism.

Method: First, thirty-two Wistar rats were randomly submitted to an acute bout of swimming exercise carrying a load of 2% ($n = 8$), 4% ($n = 8$) and 6% ($n = 8$) of their total body weight attached in their tail. Control rats remained rested ($n = 8$). Blood samples were taken from tail vein for plasma S-containing amino acids determination before (Rest) and post, 1, 2, 3, 4, 6, and 10 h after acute swimming exercise. Second, 56 exercised rats (4% loads) were euthanized before (Rest) and 1, 2, 3, 4, 6, and 10 h after acute swimming exercise. Blood and liver samples were collected for amino acids and keys genes involved in the Hcy metabolism assay.

Results: Acute exercise increases ($P < 0.05$) plasma Hcy concentration in an intensity-dependent manner (rest 7.7 ± 0.8 ; 6% load 13.8 ± 3.6 ; 4% load 12.2 ± 2.9 and 2% load 10.1 ± 2.6 , $\mu\text{mol/L}$); this increase is transient and does not promote hyperhomocysteinemia ($< 15 \mu\text{mol/L}$). Exercise-induced increased plasma Hcy was accompanied by the decreased liver S-adenosylmethionine/S-adenosylhomocysteine ratio and elevated MAT1a mRNA content. Acute exercise also caused elevated mRNA of key enzymes of transsulfuration (CBS) and remethylation (BHMT and the MTRR).

Conclusion: Our data provided evidence that acute exercise increases plasma Hcy concentration due to the augmented requirement for methylated compounds that increases liver SAM consumption. Also, Hcy remethylation and transsulfuration are coordinately regulated to maintain methyl balance.

1. Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid, a sub-product of demethylating methionine during the cellular transmethylation pathway [1]. Hcy has been studied extensively due its relationship with several chronic diseases [2]. Under normal circumstances, Hcy concentration is regulated by two pathways that either catabolize Hcy or remethylate Hcy to reform methionine [3]. Hcy catabolism occurs by transsulfuration pathway under the action of the cystathionine- β -synthase (CBS) enzyme. Alternatively, Hcy can be methylated back to methionine via methionine synthase (MS) or betaine-homocysteine S-methyltransferase (BHMT), both enzymes that transfer methyl groups from 5-methyltetrahydrofolate or betaine, respectively [3,4]. On the opposite side, an imbalanced Hcy removal via transsulfuration or re-novation via remethylation may promote elevated Hcy plasma levels that are substantially associated with numerous diseases [5] and an

increased risk of mortality [6,7].

In the last few years, studies have demonstrated exercise alters Hcy levels in the blood of rodents and humans [8–11]. Recently, Deminice et al. [9] reviewed 22 relevant studies on the effects of exercise on Hcy plasma levels and the meta-analysis demonstrated acute exercise increases plasma Hcy concentration after both low-to-moderate and high intensity acute exercise. Studies have also demonstrated exercise cause a transient increase in Hcy but not hyperhomocysteinemia ($> 15 \mu\text{mol/L}$) [9,12]. However, these authors failed to demonstrate a mechanistic explanation by which exercise elevates Hcy plasma concentration [9]. So far, mechanistic proposals have included increased protein catabolism induced by exercise, including the catabolism of amino acids involved in one-carbon metabolism as Hcy [13,14]; increased demand for vitamin B-6 and folate, vitamins involved in the catabolism and remethylation of Hcy in the liver, respectively [10]; and augmented methyl flux for methylated compound formation such as creatine and L-

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Table 1

Total body weight and acute exercise characteristics of rested and swimming rats at 2, 4 and 6% exercise load.

	Rest	2%	4%	6%
Body weight (g)	264.8 ± 18.4	256.8 ± 16.1	261.7 ± 16.6	267.5 ± 17.3
Swimming load (g)	–	5.1 ± 0.4 ^a	10.4 ± 0.6 ^{a,b}	16.0 ± 1.2 ^{a,b,c}
Post exercise blood lactate (mmol/L)	3.2 ± 0.5	6.3 ± 0.9 ^a	8.1 ± 1.4 ^{a,b}	15.1 ± 2.2 ^{a,b,c}
Swimming time (min)	0	60	60	29.5 ± 9.5 ^{b,c}

Mean values ± DP.

^a Significantly different from Rest.^b Significantly different from 2%.^c Significantly different from 4% ($P < 0.05$ by ANOVA followed by the Tukey posttest).

carnitine, compounds required during exercise [8,15,16]. However, the mechanisms involved in exercise-induced changes in Hcy plasma concentration nowadays are poorly known.

In addition, since exercise is one of the most widely recommended tool to reduce cardiovascular disease risk and elevated Hcy is an independent predictor of cardiovascular disease risk, is important to understand Hcy formation in response to different duration and intensities of exercise. It may therefore optimize exercise prescription to patients with cardiovascular disease. Important to say that studies involved in understand Hcy plasma changes induced by exercise that control duration and exercise intensity are scarce. Thus, the aim of the present study was to determine the effects of different intensities of acute exercise on Hcy plasma levels, and the exercise-induced changes in Hcy liver metabolism. Our hypothesis is that exercise increases Hcy plasma concentration in an intensity-dependent manner; and that it up-regulate transmethylation pathway in response to increased methylation flux.

2. Methods

2.1. Animals and treatment

In all, 88 male Wistar rats were obtained from the Biological Sciences Center at the State University of Londrina. All procedures were approved by the Ethics Committee for Animal Use at the same institution, and were in accordance with the Guidelines of the COBEA (Brazilian College of experiments with animals) and with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. The rats were weighed every two days and were housed in collective cages on a 12-h light/dark cycle at a mean temperature of 22 °C. The animals also had free access to food and water throughout the experiment and received fresh food and water every 2 days.

To determine the effects of different intensities of acute exercise on Hcy plasma levels and metabolism, two different experiments were conducted. First, after one-week exercise adaptation, 32 rats (initial weight ~230 g) were randomly submitted to an acute bout of swimming exercise in three different conditions: swimming with a load of 2% ($n = 8$), 4% ($n = 8$) and 6% ($n = 8$) of their total body weight attached in their tail. Rest controls ($n = 8$) remained without any exercise stimuli. Two hundred microliter blood samples were taken from a tail vein for plasma S-containing amino acids determination before (Rest) and post, 1, 2, 3, 4, 6, 10 h after acute swimming exercise. Twenty microliter blood samples at time post exercise was used to blood lactate concentration assay after different load swimming.

For the second experiment, 56 rats were adapted to swimming exercise and then submitted to an acute bout of swimming exercise with a load of 4% of their total body weight. Rats were then euthanized before (Rest) and post, 1, 2, 3, 4, 6, 10 h after acute swimming exercise. Blood and liver tissue were collected for amino acids and key genes involved in the Hcy metabolism assay.

2.2. Acute swimming exercise protocol

Acute swimming exercise was performed in both experiments in accordance with previously described by Deminice et al. [8]. Briefly, after 1 week of adaptation (progressively increasing the animal exposure to the water from 15 to 60 min at ~30 °C, 5 days/week) the animals were submitted to the acute swimming exercise protocol. The purpose of the adaptation periods was to reduce stress without promoting exercise training adaptations. The acute exercise test consisted of a maximum of 1 h of swimming carrying a load of 2, 4 or 6% of body weight (adapted elastic tape attached to the rat's tail) in a 50 cm deep water tank at ~30 °C. These loads were chosen because according to Voltarelli et al. [17], a load of 4% of body weight corresponds to a lactate threshold swimming intensity. Thus, 2, 4 or 6% of body weight loads correspond to under, at and above lactate threshold swimming exercise intensities, respectively.

2.3. Euthanasia and tissue preparation

All rats were anesthetized with an intramuscular injection of a mixture of ketamine and xylazine (65 mg/kg). After experiment 2, blood was collected from abdominal vein into heparinized tubes, centrifuged and the plasma was stored at –80 °C. A portion of the liver was freeze-clamped, weighed and stored at –80 °C. All procedures were performed under standard RNase-free conditions to avoid exogenous RNase contamination.

2.4. Homocysteine and related metabolites

Plasma Hcy as well as other related amino acids were measured by gas-chromatography (GCFID, GC-17A Shimadzu®, Kyoto, Japan) after derivatization using the commercially available kit EZ:Faast Amino Acid Analysis kit (Phenomenex®). Hepatic S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) were determined using a HPLC (Shimadzu®, Kyoto, Japan) according to Deminice et al. [18].

Blood lactate concentration was determined using a commercially available kit (Labtest®, Lagoa Santa, Brazil).

2.5. Gene expression

Total RNA was isolated from 50 mg of frozen liver using a RiboPure Kit according to the manufacturer's instructions (Ambion, part number AM 1924, USA). Total RNA was quantified using a NanoDrop2000c spectrophotometer. Then, DNase I treatment (DNA-free Kit, Ambion, part number AM1906, USA) was performed to remove contaminating DNA from isolated total RNA. cDNA was synthesized from 1000 ng of total RNA using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, part number 4374966, USA). Quantitative real-time PCR was performed using ViiA™ 7 Real-time PCR System (Applied Biosystems, USA). The following Taqman® Gene Expression Assays (Applied Biosystems, USA) were used in this study: Rn00564517_m1 (Pemt), Rn00578255_m1 (Bhmt), Rn00567215_m1 (Gnmt), Rn00560948_m1 (Cbs), Rn00563454_m1 (Mat1a, Methionine

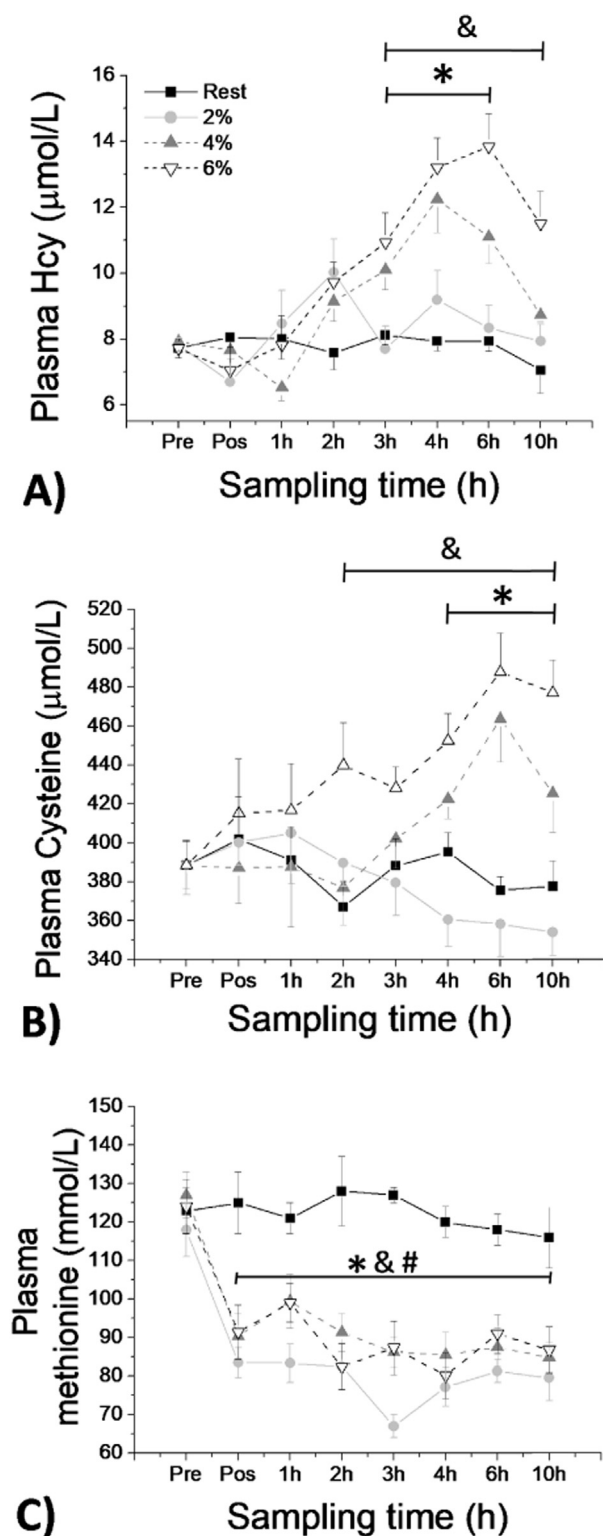


Fig. 1. Plasma Hcy (A), cysteine (B) and methionine (C) concentration determined at rest and post, 1, 2, 3, 4, 6, and 10 h after swimming exercise carrying a load of 2 (●), 4 (▲) and 6% (▼) of total body weight for rested animals (■). Mean values $\pm$ DP. *Significantly different from Rest; & significantly different from 1 h; # significantly different from 2 h; \$ significantly different from 3 h ($P < 0.05$ by linear mixed effects model).

Adenosyltransferase 1A), Rn01409369_m1 (Mtrr, Methionine synthase reductase), Rn00690933_m1 (cyclophilin A). One cycle of 95 °C for 20 s, 40 cycles of 30 s at 95 °C, and 30 s at 60 °C were used as a PCR cycles. Each PCR assay was performed in triplicate. Cyclophilin A was

used as a reference gene to normalize the reactions. The relative quantitation was determined by the $2^{-\Delta\Delta CT}$ method.

2.6. Statistical analysis

Data were reported as mean $\pm$ standard deviation. For possible differences in general and physiological characteristics between groups, one-way ANOVA with Tukey's post-test was used. A linear mixed effects model was used to detect possible differences in amino acids and related Hcy metabolites relative to different exercise intensities and sampling time. The SPSS statistical package (version 20.0) was used for statistical analysis. A P value of < 0.05 was considered to be significant in all cases.

3. Results

Table 1 presents the total body weight and physiological characteristics of rested and exercised rats at 2, 4 and 6% loads. No significant differences were demonstrated for total body weight of rats at different exercise intensities. Blood lactate concentration increased progressively ($P < 0.05$), a result of the gradual increase in swimming load. As a consequence, rats swimming with 6% load exercised half of the time when compared to 2 and 4% exercised load rats.

Plasma Hcy, cysteine and methionine concentration determined at rest and post (0 h), 1, 2, 3, 4, 6, and 10 h after acute swimming exercise with a load of 2, 4 and 6% were presented in Fig. 1. Hcy plasma concentration increased ($P < 0.05$) in an intensity-dependent manner, with 79% maximal increase at 6% exercise load, 58% maximal increase at 4% exercise load and 30% maximal increase at 2% exercise load. Time-to-peak of increase was also different among the intensities studied, demonstrating that the higher the intensity of the exercise, the later the plasma Hcy peak (6% load peak at 6 h, 4% load peak at 4 h and 2% load peak at 2 h). Intense exercise also exposed the animals to the longer times of elevated Hcy (AUC: 23.1 ± 3.9 at 6% load; 19.3 ± 3.4 at 4% load and 11.3 ± 2.4 at 2% load). In addition, elevated Hcy promoted by acute exercise demonstrated a transient behavior, since it returned to rest levels 10 h after the swimming exercise.

Cysteine plasma concentration was also increased in an intensity-dependent manner, as demonstrated for Hcy. On the opposite side, methionine plasma levels were decreased ($P < 0.05$) after acute swimming exercise compared to rest levels independent of volume and intensity of exercise; it remained diminished until 10 h after the exercise (Fig. 1).

Liver sample analysis demonstrated that acute swimming exercise promoted decreased ($P < 0.05$) liver SAM while elevating liver SAH concentration. The effects of acute exercise on liver Hcy metabolites is clearly evident in the SAM/SAH ratio that remained significantly lower ($P < 0.05$) compared to rest levels for 6 h after exercise. All these changes occurred in association with increased ($P < 0.05$) plasma Hcy concentration induced by acute exercise (Fig. 2). Plasma Hcy related amino acids were also presented in Table 2. Plasma cysteine and serine were elevated, while methionine plasma concentration was reduced after acute exercise. No change in glycine plasma concentration was imposed by acute exercise.

The mRNA expression of key genes involved in the metabolism of S-containing amino acids is presented in Fig. 3. The transmethylation gene MAT1a was significantly increased ($P < 0.05$) while GNMT was significantly decreased ($P < 0.05$) after acute exercise. The increased CβS gene expression demonstrates that acute exercise promotes elevated transsulfuration pathway flux. In the same way, acute exercise promoted elevation in the three remethylation genes analyzed, demonstrating the increasing flux to de novo formation of Hcy to methionine promoted by exercise (important to note the transient decreased MTrr gene expression in the first 2 h with posterior elevation for the next 6 h).

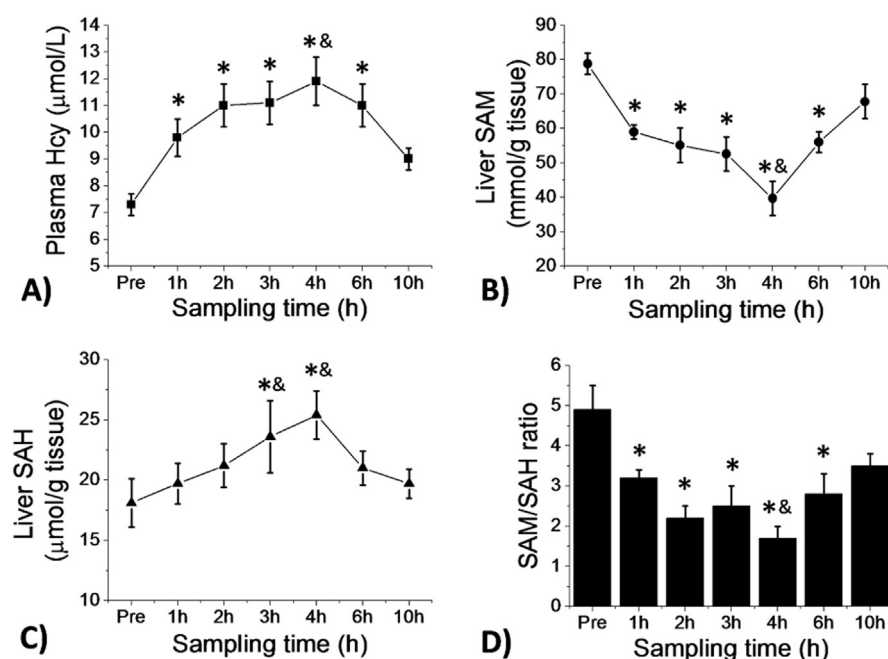


Fig. 2. Plasma Hcy (A) and liver concentration of SAM (B), SAH (C) and SAM/SAH ratio (D) determined at rest and 1, 2, 3, 4, 6, and 10 h after acute swimming exercise carrying a load of 4% of total body weight. Mean values $\pm$ DP. *Significantly different from Rest; & significantly different from 1 h ($P < 0.05$ by linear mixed effects model).

Table 2

Plasma Hcy related amino acids determined at rest and 1, 2, 3, 4, 6, and 10 h after acute swimming with an exercise load of 4%.

	Rest	1 h	2 h	3 h	4 h	6 h	10 h
Methionine (μmol/L)	103.3 $\pm$ 14.2	76.7 $\pm$ 11.5	71.7 $\pm$ 11.4 ^a	67.3 $\pm$ 14.6 ^a	70.3 $\pm$ 11.0 ^a	69.3 $\pm$ 9.9 ^a	74.6 $\pm$ 7.8 ^a
Cysteine (μmol/L)	383.3 $\pm$ 63.5	388.6 $\pm$ 90.9	418.2 $\pm$ 48.1	387.9 $\pm$ 41.4	392.9 $\pm$ 78.7	507.1 $\pm$ 65.1 ^{a,b,c,d}	532.8 $\pm$ 161.2 ^{a,b,c,d}
Serine (μmol/L)	438.9 $\pm$ 173.5	415.5 $\pm$ 106.1	562.0 $\pm$ 94.8	662.7 $\pm$ 145.4 ^{a,b}	504.1 $\pm$ 96.7	571.9 $\pm$ 93.0 ^{a,b}	493.5 $\pm$ 73.5
Glycine (μmol/L)	861.2 $\pm$ 217.9	681.4 $\pm$ 144.1	802.5 $\pm$ 154.1	786.1 $\pm$ 195.0	766.9 $\pm$ 168.1	713.2 $\pm$ 137.6	650.8 $\pm$ 1168.6

Mean values $\pm$ DP.

^a Significantly different from Rest.

^b Significantly different from 1 h.

^c Significantly different from 2 h.

^d Significantly different from 3 h ($P < 0.05$ by linear mixed effects model).

4. Discussion

The principal findings of the present study were: 1) acute exercise increased plasma Hcy concentration in an intensity-dependent manner; 2) this increase was transient and did not promote hyperhomocysteinemia; 3) exercise-induced increased plasma Hcy was probably due to the augmented requirement for methylated compounds that lead to increased liver SAM consumption; 4) acute exercise also caused elevated mRNA of key enzymes of transsulfuration (CβS) and remethylation (BHMT and the MTrR) to augment Hcy catabolism and renovation, respectively; all these in an attempt to maintain methyl balance (Fig. 4).

As demonstrated in our study (experiment 1), Hcy plasma concentration increased progressively in relation to the rise in swimming load imposed on the rats. This is in accordance with previously compiled data which demonstrated elevated Hcy plasma levels induced by acute exercise [9]; Nevertheless, to the best of our knowledge, our is the first study demonstrating that exercise increases Hcy plasma concentration in an intensity-dependent manner. Other have demonstrated no changes [19] or elevations related to exercise duration [9,12]. We also demonstrated that the increase in Hcy after acute exercise is transitory and liable to return to rest values in a few hours (~10–12 h). In addition, increased Hcy induced by acute exercise was not enough to cause hyperhomocysteinemia ($> 15 \mu\text{mol/L}$) and was trivial compared to that caused by pathological conditions such as renal chronic failure [20], dementia [21,22], and cardiovascular disease [23]. Taking these data together, we may affirm that increased Hcy induced by acute exercise may not be deemed a risk factor of cardiovascular events or any

other disease mediated by hyperhomocysteinemia [24].

Although the majority of studies demonstrated elevated plasma Hcy concentrations after acute exercise, the mechanistic explanation for this effect remains poorly investigated. Early studies have proposed that elevated amino acids pool in consequence of the protein catabolism induced by exercise [25] should lead to an increased catabolism of the intermediary metabolism and Hcy formation [13,14]. Studies have shown increased plasma and muscle free amino acids after acute exercise [25,26], demonstrating the elevated amino acids pool in consequence of physical effort. However, recent studies have demonstrated plasma methionine decreased after acute exercise [8,16], which was also demonstrated in the present study (Fig. 1). It seems contradictory since Hcy is formed exclusively by demethylation of methionine [4]. Therefore, increased pool of amino acids by acute exercise appears to not be the only thing responsible for the elevation in Hcy formation, since Hcy is formed exclusively by demethylation of methionine that is decreased after acute exercise.

Indeed, increased Hcy appears to be a consequence of the increased methyl flux imposed by acute exercise. SAM is the most important methyl donor in the liver [1,27]. Therefore, a sufficient bioavailability of SAM is required for the synthesis of methyl-compounds required during and after exercise (i.e., DNA, epinephrine, acetylcholine, carnitine, creatine) [10]. As an example, creatine phosphate is required as an immediate energy source for muscle contraction [28]. Creatine synthesis, therefore, is responsible for a considerable consumption of SAM in the liver and Hcy formation [3]. Thus, acute exercise may increase creatine synthesis elevating methylation demand and Hcy formation, as

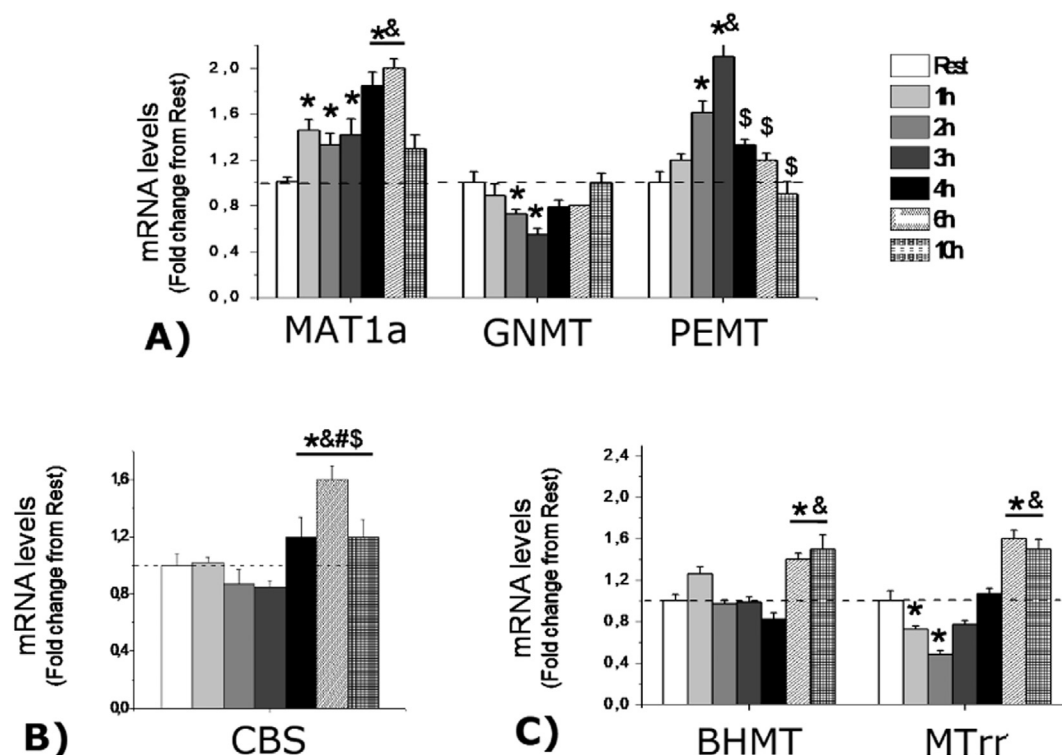


Fig. 3. mRNA levels of key genes involved in transmethylation (A); transsulfuration (B) and remethylation (C) pathways. Mean values $\pm$ DP. *Significantly different from Rest; & significantly different from 1 h; # significantly different from 2 h; \$ significantly different from 3 h ($P < 0.05$ by linear mixed effects model).

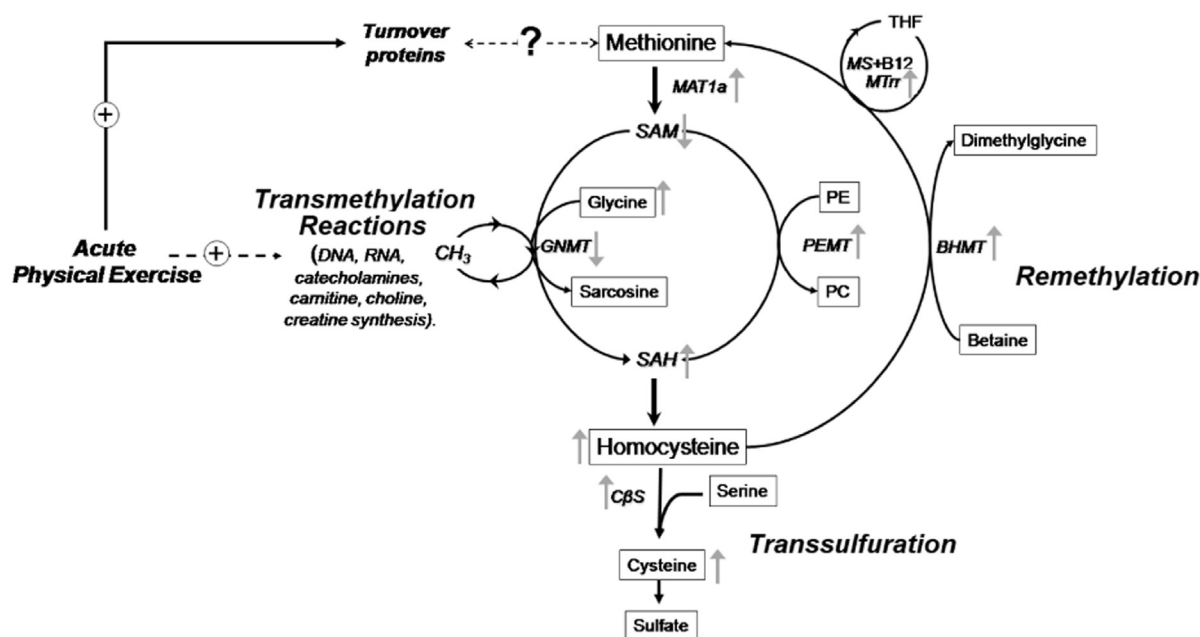


Fig. 4. Schematic representation of changes promoted by acute swimming exercise on Hcy metabolism. Acute exercise increased transmethylation reactions flux that elevated Hcy formation. As a consequence, the transsulfuration and remethylation pathways are up-regulated in an attempt to remove the excess of Hcy and increase liver SAM pool. S-adenosylmethionine (SAM); S-adenosylhomocysteine (SAH); THF, tetrahydrofolate; MS, methionine synthase; GNMT, glycine *N*-methyltransferase; CBS, cystathionine- β -synthase.

a consequence [8,15]. Phosphatidylethanolamine *N*-methyltransferase (PEMT) action for phosphatidylcholine formation in the liver is also a considerable SAM consumer (possibly the higher) through the transmethylation pathway [29]; our results demonstrated elevated PEMT mRNA levels after acute exercise. Thus, acute exercise may increase transmethylation reactions flux and SAM consumption, with a consequent elevation in Hcy formation. We confirmed acute exercise increased transmethylation reaction flux demonstrated by decreased liver

SAM and methionine plasma concentration, as well as elevated liver Mat1a gene expression. As a consequence, it increases both liver transmethylation products SAH and Hcy. The increased methyl flux is evident demonstrated by the substantial reduction in liver SAM/SAH ratio over time; this dynamic is consistent with the increased Hcy plasma concentration.

As a consequence of Hcy elevation, our results also demonstrated elevated transsulfuration pathway flux, confirmed by increased CBS

liver gene expression and plasma cysteine. In transsulfuration pathway, CβS catalyze the reaction of Hcy with serine to form cystathione, which is then hydrolyzed to form cysteine and sulfates. Thus, CβS is responsible for the catabolism of Hcy and cysteine formation [27]. CβS null mice presented severe homocysteinemia [30], while mammals fed high methionine diets presented elevated Hcy formation and CβS activity [31,32]. Indeed, the increased transsulfuration flux demonstrated in the present study is certainly an attempt to remove Hcy excess caused by acute exercise. Remethylation pathway is also an alternative way to remove Hcy excess by renovating it to methionine using new methyl groups [4,27]. We demonstrated a transient decrease followed by increased MTRr mRNA levels that added to the elevated BHMT gene expression shown increased remethylation pathway flux promoted by acute exercise. Indeed, methyl balance between transsulfuration versus remethylation is regulated both in response to Hcy concentration and the need to generate methylated compounds using cellular SAM concentration [3]. Studies on high methionine intake have demonstrated elevated Hcy plasma levels and liver SAM concentration, which facilitate transsulfuration while limiting Hcy remethylation, while a lower hepatic concentration of SAM enhances Hcy renovation via remethylation [31]. Thus, taking all our results together, it is reasonable to say that acute exercise increases the transmethylation reactions flux by increasing the demand for methylated compounds, clearly demonstrated by decreased SAM liver concentration causing increased liver SAH formation and Hcy plasma concentration. It increases the catabolism and renovation of Hcy via transsulfuration and remethylation, respectively, in an attempt to remove Hcy excess and increase the pool of liver SAM. Certainly, the absence of liver transsulfuration and remethylation enzyme activities to confirm gene expression changes induced by exercise is the principal limitation of the present study.

In conclusion, our data provided evidence that acute exercise increases plasma Hcy concentration in an intensity-dependent manner; this increase is probably due to the augmented requirement for methylated compounds that considerably increases liver SAM consumption. These changes combined with increased mRNA key genes demonstrate remethylation and transsulfuration pathways coordinately regulated Hcy flux to repair shifts in methyl balance imposed by acute exercise.

Conflict of interest

Authors declare they have no conflict of interest.

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