Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate

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A NUMBER OF EPIDEMIOLOGICAL STUDIES have confirmed a relationship between an increased plasma concentration of homocysteine and the development of vascular disease (for review, see Ref. 27). A meta-analysis performed by Boushey et al. (6) of 27 studies showed that homocysteine was an independent, graded risk factor for atherosclerotic disease. Total plasma homocysteine values of ~10 μM for men and ~8 μM for women are considered normal. However, as little as a 5-μM increase in total plasma homocysteine is associated with an increased risk of coronary artery disease of 60% for men and 80% for women. The mechanism by which homocysteine exerts pathological effects is currently unknown.

During the course of its metabolism, methionine is denylated by the enzyme S-adenosylmethionine synthase to form S-adenosylhomocysteine (SAM), the methyl donor in virtually all known biological methylation reactions (25). For example, the methylation of DNA and RNA and the conversion of glycine to sarcosine all require SAM as methyl donor. The end products of these methyltransferase reactions are a methylated substrate and S-adenosylhomocysteine (SAH), which is reversibly hydrolyzed to homocysteine and adenosine. Homocysteine has several possible fates: 1) catabolism to cysteine via the pyridoxal phosphate-dependent transsulfuration enzymes cystathionine β-synthase (CβS) and cystathionine γ-lyase (CγL) (24), 2) remethylation to methionine via cobalamin-dependent methionine synthase or betaine:homocysteine methyltransferase (12), and 3) export to the extracellular space.

It is apparent, therefore, that biological methylations and homocysteine metabolism are intimately linked and that alterations in one may affect the other. Such an effect has been documented in studies of patients with Parkinson’s Disease undergoing treatment with L-3,4-dihydroxyphenylalanine (L-Dopa). This disorder is characterized by an extreme depletion of nigro-striatal dopaminergic neurons that results in deficiencies of dopamine in the basal ganglia and of melanin in the substantia nigra. Treatment involves administration of L-Dopa alone or in combination with a peripheral decarboxylase inhibitor (9). The decarboxylation of L-Dopa in the brain alleviates the dopamine deficiency. In the course of treatment, a wasteful peripheral methylation of L-Dopa by catechol O-methyltransferase occurs with the production of 3-O-methyl-dopa, which is excreted. The result of this metabolic removal of L-Dopa is a therapy that requires very high...
doses of the drug. Consequently, methyl status is altered. Decreases in whole blood levels of SAM have been observed in L-Dopa-treated patients (9) as well as increases in plasma homocysteine in humans and rats (1, 23). It is clear that alterations in methylation demand by pharmacological agents can affect plasma homocysteine levels.

In light of this, an examination of the effects of creatine and guanidinoacetate (GAA) on homocysteine metabolism is warranted, because the methylation of GAA to creatine via GAA methyltransferase consumes more SAM than all other methylation reactions combined (34). Figure 1 describes the interorgan metabolism of GAA and creatine and their interaction with homocysteine metabolism. We undertook a series of experiments, in vivo and in vitro, to determine whether manipulation of methylation demand by the more physiological substrates, creatine and GAA, could affect homocysteine metabolism. The results described herein demonstrate that homocysteine metabolism is sensitive to the methylation demands imposed by physiological substrates.

MATERIALS AND METHODS

Animals, diets, and chemicals. Male Sprague-Dawley rats weighing between 250 and 300 g were used throughout the study. The animals were obtained from our institute’s breeding colony and were housed and treated in accordance with guidelines of the Canadian Council on Animal Care (7). Memorial University’s Institutional Animal Care Committee approved all procedures. All animals had free access to water and food. Rats were fed either chow or a 20% casein-based AIN-93 diet, designed to meet the nutritional requirements for growth of laboratory animals, for 2 wk before experimentation. Where indicated, diets were supplemented with 0.4% wt/wt creatine monohydrate or 0.36% wt/wt GAA. These levels were chosen because it has been previously shown that they downregulate the renal L-arginine:glycine amidinotransferase (21). Where diets were supplemented, an equivalent mass of cornstarch was omitted from the diet. Rats were housed at 22°C and exposed to a 12:12-h light-dark cycle, with the light cycle commencing at 0800. All experiments were performed immediately after the end of the dark cycle. All chemicals were purchased from Sigma Chemical (Oakville, ON, Canada), except where noted in the text.

Tissue preparation and analysis. Animals were anesthetized with pentobarbital sodium (65 mg/kg ip). After a midline abdominal incision, blood samples were collected from the abdominal aorta into heparinized syringes and placed on ice until plasma was separated by centrifugation (15 min, 3,700 g). Plasma was frozen at −20°C for later analysis. Kidneys and liver were rapidly removed and homogenized in ice-cold 50 mM potassium phosphate buffer (pH 6.9) with a Polytron (Brinkmann Instruments, Toronto, ON, Canada) for 20 s at 50% output. The homogenates were centrifuged at 18,000 g for 30 min at 4°C, and the supernatant was retained. All enzyme assays were carried out on this 18,000-g supernatant. All enzyme assays were demonstrated to be

**Fig. 1.** Creatine synthesis: interactions with hepatic homocysteine metabolism. The first step in creatine synthesis occurs in the kidney with the transfer of the amidino group of arginine to glycine to yield ornithine and guanidinoacetate (GAA) via L-arginine:glycine amidinotransferase (1). GAA enters the circulation for transport to muscle. S-adenosylmethionine (SAM), formed from methionine via S-adenosylmethionine synthase (2), donates its methyl group to the amidino group of GAA to yield creatine and S-adenosylhomocysteine (SAH). Most creatine is exported to the extracellular space. S-adenosylhomocysteine, however, is reversibly hydrolyzed to adenosine (not shown) and homocysteine via S-adenosylhomocysteine hydrolase (4). Homocysteine has several possible fates: 1) catabolism to cysteine via the pyridoxal phosphate-dependent transsulfuration enzymes cystathionine γ-lyase (6), 2) remethylation to methionine via cobalamin-dependent methionine synthase (7) by use of 5-methyltetrahydrofolate supplied by the 5,10-methylenetetrahydrofolate reductase reaction (8) as methyl donor or via the betaine-homocysteine S-methyltransferase (not shown), which uses betaine as methyl donor, and 3) export to the extracellular space.

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linear with time and with protein under the conditions employed.

For the assay of muscle metabolites, hindlimb skeletal muscle was freeze-clamped and stored at −70°C for later analysis. Frozen tissue, still in liquid N 2, was ground with a pestle and mortar, and the powder was homogenized in 4 volumes of 6% (wt/vol) HClO 4. Extracts were centrifuged at 14,000 × g for 15 min at 0°C. Supernatants were decanted and neutralized with 30% KOH. Muscle and plasma creatine (4), muscle phosphocreatine (20), and adenine nucleotides (16) were assayed as previously described.

Analytical procedures. The following enzymes, responsible for the remethylation of homocysteine, were measured in liver: methionine synthase (19), methylenetetrahydrofolate reductase (11), and betaine-homocysteine S-methyltransferase (32). The enzymes involved in the catabolism of methionine to cysteine were also measured: CB 5S (22) and C_Y-L (29). For betaine-homocysteine S-methyltransferase and CB 5S assays, methionine and cystathionine, respectively, were measured by HPLC (18). The l-arginine:glycine amidinotransferase was assayed in kidney using the method of Van Pilsun et al. (30), in which ornithine is detected by ninhydrin. Protein concentration was determined using the Biuret method (13) after solubilization with deoxycholate and with bovine serum albumin as a standard (17).

Total plasma homocystine concentrations were determined by reverse-phase HPLC and fluorescence detection of ammonium 7-fluoro-2-oxa-1,3-diazole-4-sulphonate (SBDF) thiol adducts by use of the method of Vester and Rasmussen (31). For plasma methionine analysis, the samples were deproteinized with 10% sulfosalicylic acid, the protein was removed by centrifugation, and the supernatant was adjusted to pH 2.2. The samples were then analyzed on a Beckman 121 MB amino acid analyzer with a Benson D-X, 14,000 × g for 15 min at 0°C. Supernatants were decanted and neutralized with 30% KOH. Muscle and plasma creatine (4), muscle phosphocreatine (20), and adenine nucleotides (16) were assayed as previously described.

Preparation of isolated hepatocytes. Hepatocytes were isolated as previously described (5), and viability was assessed by 0.2% trypan blue exclusion. Viability was ≥95% in all cases. Hepatocytes were preincubated for 20 min at 6–8 mg dry weight of cells/ml in a final volume of 1 ml in Krebs-Henseleit medium containing 1.25% bovine serum albumin. At the end of the preincubation, substrates were added, and the incubation was allowed to continue for an additional 30 min. Cells were gassed with 95% O 2-5% CO 2 at the beginning of the incubation and at the addition of substrates.

When homocysteine export was measured, the cells plus incubation medium were immediately centrifuged at 14,000 g (Brinkman Instruments, Rexdale, ON, Canada) for 2 min to sediment the cells, and an aliquot of the supernatant was removed for exported thiol analysis. This involves reduction of all thiols with 10% tri-N-butylphosphine in dimethylformamide, followed by deproteinization with perchloric acid and derivatization with SBDF (see Analytical procedures).

Statistics. Data were analyzed by ANOVA followed by the Newman-Keuls multiple comparison posttest. In all cases, P < 0.05 was taken to indicate significant difference.

Table 1. Effect of GAA and creatine supplementation on hindlimb skeletal muscle metabolites and plasma creatine

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GAA</th>
<th>Creatine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatine</td>
<td>9.6 ± 0.9a</td>
<td>13.3 ± 2.2b</td>
<td>14.0 ± 2.4a</td>
</tr>
<tr>
<td>Phosphocreatine</td>
<td>12.2 ± 2.2a</td>
<td>13.1 ± 1.1b</td>
<td>15.2 ± 2.3a</td>
</tr>
<tr>
<td>Total muscle creatine</td>
<td>21.8 ± 1.8a</td>
<td>26.4 ± 1.8b</td>
<td>29.2 ± 1.3a</td>
</tr>
<tr>
<td>Plasma creatine</td>
<td>0.06 ± 0.02a</td>
<td>0.35 ± 0.04b</td>
<td>0.31 ± 0.04b</td>
</tr>
<tr>
<td>ATP</td>
<td>5.5 ± 0.1a</td>
<td>5.4 ± 0.3b</td>
<td>6.2 ± 0.5a</td>
</tr>
<tr>
<td>ADP</td>
<td>0.96 ± 0.10a</td>
<td>0.90 ± 0.05b</td>
<td>0.96 ± 0.07a</td>
</tr>
<tr>
<td>AMP</td>
<td>0.066 ± 0.005a</td>
<td>0.091 ± 0.046b</td>
<td>0.091 ± 0.031a</td>
</tr>
</tbody>
</table>

Rats were maintained on control, guanidinoacetate (GAA)-, and creatine-supplemented 20% casein AIN-93 diets for 2 wk as described in MATERIALS AND METHODS. Muscle creatine, phosphocreatine, total creatine, and adenine nucleotides are given in values of μmol/g wet muscle weight. Plasma creatine is given as mM. Values are means ± SD; n = 5 for control and GAA-supplemented groups; n = 4 for the creatine-supplemented group. Data were analyzed by ANOVA followed by the Newman-Keuls posttest, with P < 0.05 taken to indicate significant difference. Data with differing superscripts are significantly different within rows.

RESULTS AND DISCUSSION

Effect of creatine and GAA supplementation. Table 1 describes the effect of creatine and GAA supplementation on muscle metabolites in hindlimb skeletal muscle and on plasma creatine. Muscle creatine was increased by 39% in GAA-supplemented animals and by 46% in the creatine-supplemented group compared with control values. Phosphocreatine was unchanged. Plasma creatine was about sixfold higher in both the GAA- and creatine-supplemented groups. These data indicate that dietary supplementation with creatine or GAA significantly alters both muscle and plasma creatine levels.

Table 1 also shows the adenine nucleotide content of the hindlimb skeletal muscle. No statistically significant differences in ADP or AMP were noted among groups. However, ATP was slightly elevated in the creatine-supplemented group.

Total plasma homocysteine was shown to be sensitive to dietary manipulation of methyl acceptors (Fig. 2). Animals maintained on a GAA-supplemented diet exhibited a plasma homocysteine level that was 49% higher than control animals. Supplementation of diets with creatine, however, resulted in a 27% decrease in plasma homocysteine. Plasma methionine was unchanged.

Effect of GAA and creatine supplementation on selected liver and kidney enzymes. Several enzymes of interest to homocysteine and GAA metabolism were assayed (Table 2). The two enzymes of the transsulfuration pathway, which together catalyze the catabolic removal of homocysteine from the methionine cycle, were assayed in liver. The activity of the first enzyme, CB 5S, was 89% higher in the GAA-supplemented group than in the creatine-supplemented group. However, neither group differed significantly from the control group. The activity of liver C_Y-L, the final enzyme of the transsulfuration pathway, was unchanged among groups.

Three enzymes required for the remethylation sequence of homocysteine, methionine synthase, methyl-

1“Total plasma homocystine” refers to the sum of free and protein-bound homocystine, as well as mixed disulfide forms.
Finally, the activity of \( l \)-arginine:glycine amidinotransferase, which catalyzes the synthesis of GAA, was assayed in kidney tissue. As expected, GAA and creatine supplementation resulted in a substantial down-regulation of enzyme activity. Activity in the GAA- and creatine-supplemented groups was just 27 and 18% of control values, respectively.

**Effect of GAA and creatine on homocysteine export from isolated rat hepatocytes.** To further characterize the effect of GAA and creatine on homocysteine metabolism, we undertook a series of experiments in which we incubated isolated primary rat hepatocytes in the presence and absence of GAA, creatine, methionine, and serine. The results of these experiments are shown in Table 3. In the presence of methionine, GAA significantly increased the rate of homocysteine export (47% vs. methionine alone), but creatine was without effect. When cells were incubated with methionine plus serine, homocysteine export was decreased by 40% compared with cells incubated with methionine alone. This “serine effect” was also evident in incubations containing methionine plus GAA. When serine was included in these incubations, homocysteine export was lowered by 51%, to the same level of export as the methionine plus serine incubations. These data suggest that alterations in plasma homocysteine imposed by methylation demand may be partly due to effects on liver metabolism.

The objective of this study was to investigate the effects of alterations in methylation demand imposed by GAA and creatine on homocysteine metabolism. GAA would increase methylation demand, and creatine would decrease it. As stated earlier, peripheral \( l \)-Dopa methylation by SAM has been shown to elevate plasma homocysteine (1, 23). A similar effect on plasma homocysteine has been observed by Basu and Mann (3) in a study investigating the effects of niacin supplementation on methionine metabolism in rats. Niacin has been shown to exert hypolipidemic effects.

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**Table 2. Effect of GAA and creatine supplementation on selected liver and kidney enzymes**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GAA</th>
<th>Creatine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver enzymes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystathionine β-synthase</td>
<td>5.4 ± 1.3</td>
<td>7.2 ± 1.6</td>
<td>3.8 ± 1.2</td>
</tr>
<tr>
<td>Cystathionine γ-lyase</td>
<td>10.1 ± 2.6</td>
<td>10.3 ± 0.8</td>
<td>10.8 ± 1.7</td>
</tr>
<tr>
<td>Methionine synthase</td>
<td>0.23 ± 0.02</td>
<td>0.31 ± 0.03b</td>
<td>0.23 ± 0.02a</td>
</tr>
<tr>
<td>S-methyltransferase</td>
<td>0.28 ± 0.07a</td>
<td>0.24 ± 0.03a</td>
<td>0.35 ± 0.04b</td>
</tr>
<tr>
<td><strong>Kidney enzyme</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( l )-Arginine:glycine amidinotransferase</td>
<td>1.1 ± 0.2</td>
<td>0.3 ± 0.3</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

Rats were maintained on control, GAA-, and creatine-supplemented 20% casein AIN-93 diets for 2 wk as described in MATERIALS AND METHODS. Assays were performed on liver tissue except in the case of \( l \)-arginine:glycine amidinotransferase, which was assayed on kidney tissue. All activities are expressed as nmol-mg protein \(^{-1}\) min\(^{-1}\). Values are means ± SD; \( n = 5 \) for all groups. Data were analyzed by ANOVA followed by the Newman-Keuls posttest, with \( P < 0.05 \) taken to indicate significant difference. Data with differing superscripts are significantly different within rows.

**Table 3. Homocysteine export from isolated rat hepatocytes**

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Homocysteine Export, nmol/mg dry wt (^{-1}) h(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine</td>
<td>2.17 ± 0.17a</td>
</tr>
<tr>
<td>Methionine + GAA</td>
<td>3.19 ± 0.22b</td>
</tr>
<tr>
<td>Methionine + creatine</td>
<td>2.08 ± 0.23a</td>
</tr>
<tr>
<td>Methionine + serine</td>
<td>1.29 ± 0.23c</td>
</tr>
<tr>
<td>Methionine + serine + GAA</td>
<td>1.56 ± 0.33c</td>
</tr>
</tbody>
</table>

Cells were isolated as described in MATERIALS AND METHODS and incubated in the presence of the substrates indicated above (all concentrations are 1 mM). Cells were preincubated for 20 min in the presence of medium alone. At the end of the preincubation period, substrates (e.g., methionine) were added, and incubations were allowed to continue for an additional 30 min, after which cells were decanted into Eppendorf tubes and centrifuged for \( \sim 90 \) s. The cell-free supernatant was removed and saved for homocysteine analysis, as described in MATERIALS AND METHODS. Values are means ± SD; \( n = 7 \) for Met and Met + Ser; \( n = 5 \) for all others. Data were analyzed by ANOVA followed by the Newman-Keuls posttest, with \( P < 0.05 \) taken to indicate significant difference. Data with differing superscripts are significantly different from each other.
when administered in pharmacological doses (10, 15). However, niacin is excreted as methylated pyridones, the formation of which uses SAM as the methyl donor. In their study, Basu and Mann maintained rats on diets supplemented with niacin and monitored effects on plasma sulfur amino acids. Their experiments showed that niacin supplementation significantly increased plasma homocysteine levels. These results, and those of Miller et al. (23) and Allain et al. (1), underscore the importance of peripheral methylation reactions, and the consequent effects on methionine metabolism, during the course of drug therapy.

Creatine biosynthesis begins with the synthesis of GAA in the kidney. L-Arginine:glycine amidinotransferase catalyzes the transfer of the amidino group of arginine to glycine, yielding ornithine and GAA (33). GAA is carried in the blood to the liver, where it is methylated by SAM via guanidinoacetate N-methyltransferase to form creatine, which is then exported to extrahepatic tissues. Creatine and creatine phosphate are converted nonenzymatically to creatinine, the excreted form. This loss has been estimated to be ~2 g/day in a 70-kg male (2). Consequently, creatine synthesis must replace that lost as creatinine, and it has been calculated that creatine biosynthesis consumes ~70% of physiologically labile methyl groups, more than all other methylation reactions combined (34).

The principal regulatory site, and rate-limiting step, in the creatine biosynthetic pathway is the L-arginine:glycine amidinotransferase reaction. Creatine exerts feedback repression on L-arginine:glycine amidinotransferase at the pretranslational level, an example of end-product repression (21). This repression permits conservation of the essential amino acids methionine and arginine. Guanidinoacetate N-methyltransferase, however, is not subject to this kind of regulation by creatine but rather is dependent only on substrate availability. In light of this, the effect of exogenous creatine would be observed after a period of only days. The effect of creatine under these conditions would be to limit the supply of GAA available for methylation by guanidinoacetate N-methyltransferase, thereby decreasing the amount of SAM converted to SAH and subsequently to homocysteine. These expectations were borne out by experiment, in that dietary creatine supplementation downregulated L-arginine:glycine amidinotransferase (Table 2) and decreased plasma homocysteine concentration (Fig. 2). As expected, addition of creatine to hepatocyte incubations had no effect on homocysteine production (Table 3).

We also predicted that provision of GAA would drive creatine synthesis and that the resultant methylation demand would increase plasma homocysteine. Dietary GAA also downregulated the renal transamidinase, presumably via the increased plasma creatine concentration. However, the decreased renal synthesis of GAA is more than offset by the provision of the dietary source of this compound. The increased provision of GAA directly drives increased homocysteine production, as is evident in the experiments with hepatocytes (Table 3). The accumulation of homocysteine in these hepatocyte experiments reflects the fact that the production of this metabolite exceeds its rate of removal by remethylation and transsulfuration reactions. We have previously shown that hepatic homocysteine accumulation is decreased by the provision of serine (28).

Serine can affect homocysteine metabolism by acting as cosubstrate for the CβS reaction (Fig. 1, reaction 5) and by providing folate-linked one-carbon units, which ultimately provide the methyl donor in the methionine synthase reaction (Fig. 1, reaction 7). In light of this, the homocysteine-lowering effect of serine observed in our hepatocyte experiments could be mediated either through enhanced transsulfuration, remethylation, or some combination. Our previous work, however, suggests that, in our hepatocyte model, provision of serine stimulates transsulfuration. At that time we sought to determine the locus of serine action by incubating hepatocytes with L-[1-14C]methionine, which when metabolized through the transsulfuration pathway will give rise to α-[1-14C]ketobutyrate (Fig. 1). α-[1-14C]ketobutyrate can be metabolized via pyruvate dehydrogenase to yield 14CO2. Label in unmetabolized α-[1-14C]ketobutyrate can be released by H2O2. Our previous experiments showed that 14CO2 was significantly higher in hepatocytes incubated with 1 mM L-[1-14C]methionine and 1 mM serine than in those incubated with L-[1-14C]methionine alone. The increase in 14CO2 production was roughly equal to the decrease in homocysteine export seen under the same conditions. These results suggested that the inclusion of serine, in our hepatocyte model, acts at the level of CβS to remove homocysteine. Given that we employed identical conditions in our present work, we believe the homocysteine-lowering effect of serine observed here is due to the provision of the cosubstrate for CβS, the first enzyme in the transsulfuration pathway. The provision of serine also decreases the increased homocysteine production brought about by GAA. This confirms the view that homocysteine accumulation is due to an imbalance between its production and removal. Increased production, brought about by GAA, results in homocysteine accumulation that can be corrected by promoting the removal mechanism.

We are aware of no data on the effect of creatine supplementation on homocysteine metabolism in humans. Creatine is a popular supplement for athletes and bodybuilders and is reported by some to be effective in improving high-intensity exercise performance by enhancing ATP resynthesis as a consequence of increased phosphocreatine availability (8, 14).

It is of interest to compare the creatine ingestion by our rats with the doses typically consumed by humans. Creatine consumption by humans usually involves “loading doses” of 20 g/day for 5 days, followed by a 5 g/day maintenance dose. For a 70-kg person, this amounts to a loading dose of ~0.3 g/kg body wt and a maintenance dose of ~0.1 g/kg body wt. Our rats consumed ~10 g food·day−1·100 g body wt−1. Because the supplemented food contains 0.4% creatine, this amounts to an intake of ~0.4 g/kg body wt. One must take care in making such comparisons between ani-
mals of such different body weights because of the scaling effect of metabolism (26). We would anticipate that creatine ingestion by humans would interact with homocysteine metabolism in the same manner as it does in rats, but whether or not this would result in a significant decrease in plasma homocysteine has yet to be determined.

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