Creatine synthesis: production of guanidinoacetate by the rat and human kidney in vivo

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1Department of Biochemistry, Memorial University of Newfoundland, St. John’s, NL, Canada; 2Department of Endocrinology, Carl T. Hayden Veterans Affairs Medical Center, Phoenix; and 3Center of Metabolic Biology, Department of Kinesiology, Arizona State University, Tempe, Arizona

Submitted 30 July 2007; accepted in final form 3 October 2007

Edison EE, Brosnan ME, Meyer C, Brosnan JT. Creatine synthesis: production of guanidinoacetate by the rat and human kidney in vivo. Am J Physiol Renal Physiol 293: F1799–F1804, 2007. First published October 10, 2007; doi:10.1152/ajprenal.00356.2007.—A fraction of the body’s creatine and creatine phosphate spontaneously degrades to creatinine, which is excreted by the kidneys. In humans, this amounts to ~1–2 g/day and demands a comparable rate of de novo creatine synthesis. This is a two-step process in which L-arginine:glycine amidinotransferase (AGAT) catalyzes the conversion of glycine and arginine to ornithine and guanidinoacetate (GAA); guanidinoacetate methyltransferase (GAMT) then catalyzes the S-adenosylmethionine-dependent methylation of GAA to creatine. AGAT is found in the kidney and GAMT in the liver, which implies an interorgan movement of GAA from the kidney to the liver. We studied the renal production of this metabolite in both rats and humans. In control rats, [GAA] was 5.9 μM in arterial plasma and 10.9 μM in renal venous plasma for a renal arteriovenous (A-V) difference of ~5.0 μM. In the infusion study, arginine or citrulline markedly increased renal GAA production but infusion of glycine did not. Rats fed 0.4% creatine in their diet had increased renal A-V difference of GAA of 1.5 M in arterial plasma and 10.9 μM, and a decreased renal A-V difference for GAA of ~0.9 μM. In humans, [GAA] was 2.4 μM in arterial plasma, with a renal A-V difference of ~1.1 μM. These studies show, for the first time, that GAA is produced by both rat and human kidneys in vivo.

L-arginine:glycine amidinotransferase; transamidinase; amino acid metabolism

CREATINE AND CREATINE PHOSPHATE act to buffer the cytosolic ATP/ADP ratio in tissues that have high and variable rates of ATP usage (e.g., skeletal and cardiac muscle). Creatine kinase catalyzes the reversible transfer of the γ-phosphate group of ATP to the guanidino group of creatine to yield ADP and creatine phosphate.

ATP + Creatine ↔ ADP + Creatine Phosphate (Equation 1)

In skeletal muscle, creatine kinase activity is high, keeping its reaction at near-equilibrium. This keeps the ADP and ATP concentrations fairly constant and buffers the cytosolic phosphorylation potential (18, 27, 28). Energy storage and transmission by the creatine kinase system are hypothesized to work in two ways: as a temporal buffer and as a spatial buffer. The temporal energy buffer theory is best exemplified during episodes of high-energy use, such as muscle contraction. As soon as ATP is hydrolyzed, it must be replenished. The high-energy phosphate of creatine phosphate is transferred to ADP to regenerate ATP. This leads to an accumulation of creatine that must be rephosphorylated during recovery from exercise. The spatial energy buffer theory implies that creatine phosphate acts as an energy carrier, working to transport high-energy phosphate from sites of synthesis (mitochondria) to sites of ATP utilization in the cytosol (27). There is a need for creatine replacement due to the spontaneous, irreversible conversion of creatine and creatine phosphate to creatinine (3). Creatinine is not used by the body and is excreted in the urine. Approximately 1.7% of the body’s total creatine pool (creatine and creatine phosphate) is converted to creatinine per day (28). In a young 70-kg male, this amounts to a loss of ~2 g of creatine per day (21). This creatine has to be replenished by the dietary intake of creatine and by de novo creatine synthesis.

De novo creatine synthesis is a very simple process, involving just two enzymes. The first enzyme is L-arginine:glycine amidinotransferase (AGAT). AGAT catalyzes the transfer of a guanidino group from arginine to glycine to form ornithine and guanidinoacetate (GAA) (26). This is believed to be the regulated step of creatine biosynthesis. The second enzyme in the pathway is guanidinoacetate methyltransferase (GAMT). This enzyme catalyzes the transfer of a methyl group from S-adenosylmethionine (SAM) to GAA to form S-adenosylhomocysteine (SAH) and creatine (26). There are high activities of AGAT in the kidneys and of GAMT in the livers of various species (26). Such tissue enrichment has suggested that creatine synthesis is an interorgan process whereby GAA, produced by the kidney, is released into the circulation and is methylated to creatine in the liver. Creatine is then released from the liver and into the circulation where it can be taken up, via a specific transporter, by various tissues. However, this hypothesis has not been tested in vivo.

The regulation of creatine biosynthesis is usually thought to occur at the level of AGAT (26). An increase in serum levels of creatine results in a decrease in AGAT enzyme activity, enzyme level, and mRNA expression in rat kidney (14).

In the current study, we investigate the production of GAA, in vivo, by human and rat kidneys. We also used the rat to study the regulation of renal GAA production. We investigated the effects of creatine supply as well as substrate supply on renal GAA production. This study provided novel insights into the acute and chronic regulation of creatine synthesis.

MATERIALS AND METHODS

Chemicals and other reagents. Chemicals and the Hypersil ODS C18 reverse-phase HPLC column were purchased from Sigma

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morning, an infusion of para-injection of a small amount of iodinated contrast material. At 6:30 AM, a renal measurement of renal blood flow. Between 8 and 9 AM, a renal until completion of the experiment. At between 6:30 and 8 PM after which subjects were required to fast kcal/kg; 50% carbohydrate, 35% fat, 15% protein) was consumed on the evening before experimentation. A standard meal (10 described (16). Briefly, subjects were admitted to the University of Rochester General Clinical Research Center between 6 and 7 PM on the evening before experimentation. A standard meal (10 kcal/kg; 50% carbohydrate, 35% fat, 15% protein) was consumed between 6:30 and 8 PM after which subjects were required to fast until completion of the experiment. At ~8 AM the following morning, an infusion of para-aminohippuric acid was started for measurement of renal blood flow. Between 8 and 9 AM, a renal vein was catheterized, under fluoroscopy, through the right femoral vein and the position of the catheter tip was ascertained by injection of a small amount of iodinated contrast material. At ~9 AM, a dorsal hand vein was cannulated and kept in a thermoregulated Plexiglas box at 65°C for sampling arterialized venous blood. Starting at ~10 AM, three blood samples were obtained simultaneously from the dorsal hand vein for determination of GAA and creatinine concentrations.

Animals. The study was conducted in accordance with the Guide lines of the Canadian Council on Animal Care and was approved by Memorial University of Newfoundland’s Institutional Animal Care Committee. Male Sprague-Dawley rats were purchased from Memo rial University of Newfoundland’s breeding colony and were fed ad libitum and had free access to water. The control diet, AIN-93G (17), is creatine-free while the creatine supplementation diet was a modified version of it. For the creatine supplementation diet, 4.0 g/kg creatine monohydrate replaced 4.0 g/kg cornstarch. Rats were placed on these diets for a period of 2 wk. Before the initiation of each experimental period, animals were allowed to become accustomed to the AIN-93G powdered diet for a period of ~3-5 days. At the beginning of the feeding study, rats weighed ~200 g; at the end of the feeding period, control rats and creatine-fed rats weighed an average of ~400 g. All animals were exposed to a 12:12-h light-dark cycle, with the light cycle commencing at 8 AM. All procedures were conducted during the 12-h light cycle.

Tissue preparation. Animals were anesthetized with 65 mg/kg pentobarbital sodium ip. Following a midline abdominal incision and opening of the abdominal cavity, the renal vein was cleaned of connective tissue and blood flow was measured using a T206 small animal blood flowmeter (Transonic Systems, Ithaca, NY). Arterial and venous blood samples were taken across the kidney. Kidneys were removed and immediately placed in ice-cold 50 mM potassium phosphate buffer (pH 7.4), except for a small sample that was retained for RT-PCR analysis.

Infusion experiments. The femoral vein was cleaned to allow delivery of the 0.8-ml priming injection and the jugular vein was cannulated with PE50 tubing to allow delivery of infusate. A tongue restrainer was placed in the animal’s mouth to prevent suffocation. The abdominal cavity was then opened, the T206 probe was placed on the renal vein, the priming injection was given, and the infusion pump was set to deliver 0.037 ml/min through the jugular vein for 20 min. Blood flow was measured during the entire 20-min infusion. At 20 min, both arterial and venous blood samples were taken across the kidney. Priming injections for the different experimental conditions were 0.8 ml of either 0.9% NaCl, 200 mM arginine, 100 mM citrulline, 200 mM glycine, or 200 mM alanine. Infusions, for each condition, were either 0.9% NaCl 60 mM arginine, 30 mM citrulline, 60 mM glycine, or 60 mM alanine, respectively. All amino acid solutions were made isotonic with saline.

Creatine, GAA, creatinine, and AGAT assays. For creatine measurement, aliquots of the plasma samples were deproteinized by adding 50% vol/vol 0.6 M perchloric acid, kept on ice for 20 min, and centrifuged at 20,000 g for 5 min to sediment proteins. The supernatant was neutralized with 20% potassium hydroxide and creatine was measured as described by Lowry and Passonneau (12). For GAA measurement, the plasma samples were diluted fourfold with PBS and centrifuged through a Centricron spin filter at 10,000 g for 30 min to remove large proteins. Filtered plasma was used for GAA analysis via HPLC according to the method of Carducci et al. (6). Creatinine was measured by Clinical Laboratories, Health Sciences Center (St. John’s, NL, Canada). Renal AGAT activity was measured as described by Van Pilsum et al. (25).

RT-PCR analysis of AGAT mRNA. RNA was prepared from the kidney samples using a rapid guanidinium thiocyanate method as described by Chomczynski and Sacchi (7). Two micrograms of RNA were reverse transcribed using a one-step reverse transcription kit and amplified by 21 cycles. An upstream primer (5’-ATGCTAGCGGT- GCAGTGT-3’) and downstream primer (5’-ATAATTGACCTG- TATGCGCGG-3’) were designed from the rat kidney AGAT sequence. The primers were used to amplify a 599-bp PCR fragment. A 768-bp fragment of rat β-actin gene was coamplified using amplimer set primers. PCR products were separated on a 1.5% agarose gel. Ethidium bromide-stained gels were visualized by UV illumination and quantified by densitometry.

Amino acid analysis. Plasma samples were deproteinized by mixing 200 μl plasma, 200 μl internal standard (norleucine), and 1.0 ml 5% trifluoroacetic acid in methanol. Samples were centrifuged at 20,000 g for 5 min so as to sediment proteins. The supernatant was poured off, frozen in liquid nitrogen, and freeze-dried overnight. Amino acid analysis was as described by Bidlingmeyer et al. (2).

Statistical analysis. All data are presented as means ± SD. In studies with only two groups, Student’s paired or unpaired (depending on appropriateness) t-test was used to identify significant differences. One-way ANOVA followed by Newman-Keuls post hoc tests was used in studies involving three or more experimental groups. A P value of <0.05 was taken as significant. GraphPad Software Prism 3.0 was used for all statistical analyses.

RESULTS

Renal GAA production and its chronic regulation. Figure 1A shows that rat kidneys produce GAA and release it into the renal vein; on the control diet, GAA concentration in renal venous plasma was ~9 μM, compared with 5 μM in arterial plasma. Creatine supplementation for 2 wk massively decreased both the arterial GAA concentrations (by 72%) and renal arteriovenous (A-V) differences (by 81%). Renal AGAT activity was decreased by 86% upon creatine supplementation (Fig. 1B). Densitometric quantitation of renal AGAT mRNA, when normalized for β-actin, shows a 41% decrease in its abundance upon feeding creatine.

So as to determine the extent to which renal GAA production could account for total creatine synthesis, we measured, simultaneously, the A-V differences for GAA and creatinine across rat and human kidneys. This is based on the fact that creatinine clearance via the kidneys reflects creatine loss by conversion to creatinine and, therefore, of the need for creatine
replacement. There was no significant difference between renal GAA production and creatinine loss in rats (Fig. 2). In humans, however, renal GAA production only amounted to $\approx 10\%$ of renal creatinine excretion under both fed and fasted conditions (Table 1).

**Acute regulation of renal GAA production by substrate concentrations.** We examined the effects of acute increases in the amino acid substrates for GAA synthesis. We employed two control groups, rats infused either with NaCl or with alanine. The experimental groups were infused with glycine or arginine. We also infused rats with citrulline, which is converted to arginine in kidneys (9). We measured renal GAA production, by measuring both renal plasma flow and A-V differences, after 20 min of infusion. Glycine infusion increased the circulating levels of this amino acid by $\approx 50\%$ (Table 2) but there was no increase in renal GAA production (Fig. 3). Arginine infusion increased the plasma levels of this amino acid by $\approx 300\%$ (Table 2); renal GAA production was increased by $\approx 150\%$ (Fig. 3). Citrulline infusion, which did not increase plasma arginine levels (Table 2), almost doubled renal GAA production (Fig. 3).

**DISCUSSION**

The present study makes a number of novel contributions to our knowledge of creatine synthesis and its regulation: 1) the rat kidney produces GAA, in vivo; 2) renal GAA production, by measuring both renal plasma flow and A-V differences, after 20 min of infusion. Glycine infusion increased the circulating levels of this amino acid by $\approx 50\%$ (Table 2) but there was no increase in renal GAA production (Fig. 3). Arginine infusion increased the plasma levels of this amino acid by $\approx 300\%$ (Table 2); renal GAA production was increased by $\approx 150\%$ (Fig. 3). Citrulline infusion, which did not increase plasma arginine levels (Table 2), almost doubled renal GAA production (Fig. 3).

**Table 1. Renal metabolism of GAA and creatinine in humans**

<table>
<thead>
<tr>
<th></th>
<th>GAA</th>
<th>Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial plasma concentration, µM</td>
<td>2.4 (0.8)</td>
<td>76 (13)</td>
</tr>
<tr>
<td>Renal A-V difference, µM</td>
<td>$-1.5 (0.8)^*</td>
<td>14 (8.1)^*</td>
</tr>
<tr>
<td>Renal flux, µmol/min</td>
<td>$-1.1 (0.5)$</td>
<td>10 (5.2)</td>
</tr>
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</table>

All values are expressed as mean (SD) of 7 observations. *Arteriovenous (A-V) difference is significantly different from 0 ($P < 0.01$). GAA, guanidinoacetate.
in vivo, responds acutely to increased arginine levels but not to increased glycine levels; 3) citrulline, converted to arginine in the renal tubules, is immediately available for GAA synthesis; however, creatine ingestion does not downregulate intestinal citrulline production; 4) creatine ingestion markedly decreases renal GAA production in the rat; 5) in rats fed a creatine-free diet, renal GAA production in vivo equals creatinine loss in the urine; and 6) human kidneys also produce GAA but, unlike in the rat, this is not the major source of GAA for creatine synthesis. In addition, we confirm that creatine ingestion decreases both renal AGAT activity and mRNA level.

Since creatinine excretion is a measure of creatine and creatine phosphate breakdown and therefore, of the need for creatine replacement, our data on the equivalence of renal GAA production and creatinine excretion indicate that the kidney is the major organ for the production of GAA in the rat. Borsook and Dubnoff (4) pointed out that the activity of renal AGAT is more than sufficient to supply the body’s creatine need. However, measurements of enzyme activity under optimal conditions cannot be extrapolated to the in vivo situation; the present data provide definitive information on the in vivo situation. It cannot, however, be concluded that the kidney is the sole site of GAA production in the rat. AGAT activity has been reported in rat kidney, pancreas, brain, spleen, and testes (24). Horner (11) studied transamidination in nephrectomized rats and found that GAA synthesis was not entirely ablated; indeed, extrarenal transamidination, in this situation, may be considerable. Although we showed that the rat kidney is the major organ involved in GAA production in vivo, extrarenal transamidination may be important in terms of local roles within tissues or in times of renal dysfunction.

The situation in humans, however, is quite different as renal GAA production accounts for only ~11–12% of daily creatine loss as measured by creatinine excretion. Thus, almost 90% of daily creatine replenishment occurs without renal involvement. As our human subjects were not on a creatine-free diet, this creatine replenishment occurs via a combination of diet and de novo synthesis. Stead et al. (21) estimated that in 20- to 59-yr-olds on a typical Western diet, ~50% of the creatine need can be provided by the diet. If we take this into consideration, renal GAA production in a human may account for ~20% of total GAA synthesis. The kidney, therefore, does not appear to be the major organ involved in GAA production in humans, although it clearly plays a role. An earlier study by Sandberg et al. (19) also reported GAA production by human kidneys, in vivo. Again, GAA production amounted to only 25% of the renal creatinine loss. Although these data agree, in general, with our results, it should be appreciated that the GAA concentrations reported in this 1953 study are probably not reliable as they are fivefold those found by modern analytical techniques (8).

We report that feeding a 0.4% creatine diet for a period of 2 wk causes a downregulation of renal AGAT activity and mRNA expression as well as a decrease in in vivo renal GAA production in the rat. The downregulation of AGAT activity by creatine and GAA feeding has been previously shown by Van Pilsum (23) and in our lab by Stead et al. (20). The effect of GAA is believed to be due to the conversion of GAA to creatine and the action of creatine to downregulate the enzyme. Our findings on the decreased levels of renal AGAT mRNA brought about by creatine feeding confirm those of McGuire et al. (14). These data suggest that AGAT activity may be regulated at the pretranslational level. However, the decrease in renal AGAT mRNA expression is not as large as the decrease in renal AGAT activity (41 vs. 86%, respectively) suggesting that an additional mechanism may play a role.

We also report, for the first time, that feeding a creatine-containing diet decreases GAA production by rat kidneys, in vivo. Intake of a diet containing 0.4% creatine decreases renal GAA production by 81% and lowers plasma GAA values by 72%. A recent study conducted by Derave et al. (8) showed that feeding creatine to humans for a period of 20 wk lowered plasma GAA levels in a dose-dependent manner: by 50% during the loading phase (20 g creatine/day) and by 30% during the maintenance phase (5 g creatine/day).

Our experiments also shed light on the acute regulation of renal GAA synthesis by increased provision of the amino acid substrates, glycine and arginine. Increased provision of glycine had no effect on GAA production, whereas provision of arginine did. These experiments are best interpreted in terms of the

Table 2. Plasma amino acid levels following infusion of various substrates in rats

<table>
<thead>
<tr>
<th>Infusion</th>
<th>Glycine, μM</th>
<th>Arginine, μM</th>
<th>Citrulline, μM</th>
<th>Alanine, μM</th>
<th>Methionine, μM</th>
<th>Ornithine, μM</th>
</tr>
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<tbody>
<tr>
<td>NaCl</td>
<td>268 (66)</td>
<td>111 (51)</td>
<td>85 (31)</td>
<td>566 (133)</td>
<td>42 (9)</td>
<td>93 (21)</td>
</tr>
<tr>
<td>Glycine</td>
<td>413 (29)*</td>
<td>79 (28)</td>
<td>71 (17)</td>
<td>482 (46)</td>
<td>44 (22)</td>
<td>95 (30)</td>
</tr>
<tr>
<td>Arginine</td>
<td>235 (44)</td>
<td>433 (219)*</td>
<td>92 (21)</td>
<td>516 (67)</td>
<td>40 (23)</td>
<td>315 (134)*</td>
</tr>
<tr>
<td>Citrulline</td>
<td>244 (50)</td>
<td>98 (77)</td>
<td>347 (201)*</td>
<td>424 (58)</td>
<td>33 (9)</td>
<td>154 (53)</td>
</tr>
<tr>
<td>Alanine</td>
<td>335 (84)*</td>
<td>93 (70)</td>
<td>101 (18)*</td>
<td>925 (133)*</td>
<td>52 (10)</td>
<td>91 (33)</td>
</tr>
</tbody>
</table>

All values are expressed as mean (SD) of 6 observations. *Statistical differences from the saline control in each vertical column (P < 0.05).
kinetic properties of AGAT and the renal concentrations of the substrates. The $K_m$ of the rat kidney AGAT for glycine is reported to be 2.4–2.8 mM; that for arginine is 3.0–3.1 mM (15). We reported the rat renal levels of glycine and arginine to be 1,320 and 170 nmol/g, respectively (5); this translates to average intracellular concentrations of ~2.5 and 0.32 mM, respectively, for glycine and arginine. Given that the cell glycine concentration is close to AGAT’s $K_m$ and that the infusion only raised the circulating glycine concentration by ~50%, one may expect, at most, an increase of ~25% in GAA production. An increase of this magnitude was observed but was not statistically significant. On the other hand, cellular arginine concentration is well below the $K_m$ value of AGAT for this amino acid. The infusion increased circulating arginine concentration fourfold so that, assuming that changes in cellular arginine concentration parallel the plasma levels and applying the Michaelis-Menten equation, we may anticipate an increased GAA production of about threefold. This is remarkably close to the 2.7-fold increase that we observed. The close agreement between the observed and calculated rates may be, to some degree, fortuitous given the nature of the assumptions we were obliged to make. Nevertheless, it does serve to highlight the sensitivity of renal GAA synthesis to ambient arginine concentration. That GAA synthesis in humans is also markedly sensitive to arginine concentration is suggested by a report of GAA synthesis in children with urea cycle defects that may be normalized by the provision of arginine (1).

Finally, we draw attention to our finding that renal GAA production was doubled by the infusion of citrulline even though there was no increase in circulating arginine concentration. This result is attributable to the conversion of citrulline to arginine by the renal tubules. We already showed that this process is very sensitive to circulating citrulline concentration (23). Argininosuccinate synthetase, the enzyme that catalyzes the first committed step in arginine synthesis, is remarkably insensitive to citrulline concentration. This result is attributable to the conversion of citrulline to arginine by the renal tubules. We already showed that this process is very sensitive to circulating citrulline concentration in the rat (9). Renal arginine synthesis occurs in the cells of the proximal tubule (10) as does GAA synthesis (13, 22). Therefore, arginine synthesized in these cells becomes immediately available as a substrate for GAA synthesis. This indicates that citrulline synthesis (which occurs in the gut) may contribute, in part, to creatine synthesis. It also suggests that gut citrulline synthesis may be regulated by ingestion of creatine. To test this hypothesis, we measured gut citrulline flux in rats fed the 0.4% creatine diet. We found, however, that the need for creatine synthesis does not drive intestinal citrulline synthesis, which was not altered in the creatine-fed rats (data not shown).

In summary, this study showed, for the first time, that GAA is produced by rat kidneys, in vivo. We also showed that the human kidney produces GAA. Creatine feeding downregulates renal AGAT activity and mRNA expression as well as in vivo production of GAA by the rat kidney. Provision of the substrate glycine was without effect on renal GAA production in vivo; however, provision of the substrate arginine or citrulline, which is converted to arginine in the kidney, increased renal GAA production in the rat.

ACKNOWLEDGMENTS

The authors thank B. Hall for assistance with the infusion experiments and Dr. E. Randell for assistance with creatinine measurement.

GRANTS

This work was supported by grants from the Canadian Institutes of Health Research. This material is based on work supported in part by the Office of Research and Development, Department of Veterans Affairs.

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