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Whole Body Creatine and Protein Kinetics in Healthy Men and Women: Effects of creatine and amino acid supplementation

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Abstract

Creatine kinetics were measured in young healthy subjects, eight males and seven females, age 20–30 years, after an overnight fast on creatine free diet. Whole body turnover of glycine and its appearance in creatine was quantified using [1-¹³C] glycine and the rate of protein turnover was quantified using L-ring [²H₅] phenylalanine. The creatine pool size was estimated by the dilution of a bolus [²H₃] creatine. Studies were repeated following a five days supplement creatine 21g.day⁻¹ and following supplement amino acids 14.3 g.day⁻¹. Creatine caused a ten folds increase in the plasma concentration of creatine and a 50% decrease in the concentration of guanidinoacetic acid. Plasma amino acids profile showed a significant decrease in glycine, glutamine and taurine and a significant increase in citrulline, valine, lysine and cysteine. There was a significant decrease in the rate of appearance of glycine, suggesting a decrease in de-novo synthesis (p=0.006). The fractional and absolute rate of synthesis of creatine was significantly decreased by supplemental creatine. Amino acid supplement had no impact on any of the parameters. Creatine supplement caused a significant decrease in the rate of synthesis of creatine. This is the first detailed analysis of creatine kinetics and the effects of creatine supplement in healthy young men and women. These methods can be applied for the analysis of creatine kinetics in different physiological states.

Keywords

Creatine; Guanidinoacetic acid; Glycine; Phenylalanine; Stable isotopes; Kinetics

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Introduction

Creatine, an ergogenic compound, is an important intermediate in the metabolism of the muscle, brain, and other tissues with high energy demand and fluxes (Wyss and Kaddurah-Daouk 2000; Brosnan and Brosnan 2007). It plays a critical role in buffering and translocation of energy via the creatine kinase phosphocreatine system. Functional impairment of this system leads to deterioration of energy metabolism, which is the phenotype for many neurodegenerative and age related disorders. It has been shown to affect satellite cell proliferation and differentiation in cell culture systems (Vierck et al. 2003), and increase satellite cell mitotic activity during compensatory hypertrophy in the rat skeletal muscle (Dangott et al.2000). In humans, creatine supplementation, in combination with strength training, amplified the training induced increase in satellite cell number and myonuclei concentration in skeletal muscle fibers (Olsen et al 2006). A beneficial effect of creatine supplementation on muscle performance during resistance training has been suggested in some studies. Quantification of protein turnover following creatine supplementation has shown either no effect or a decrease in protein breakdown (Louis et al 2003). However supplement did not appear to impact the fractional rate of protein synthesis in the skeletal muscle.

Creatine is synthesized by a two-step process from glycine, arginine and methionine. The first step involves the synthesis of guanidinoacetic acid (GAA) from glycine and arginine catalyzed by the reversible arginine glycine amidinotransferase (AGAT, EC2.1.4.1). Irreversible methylation of GAA by guanidine N-methyltransferase (GNMT, EC 3.5.3.2) utilizes s-adenosylmethionine (SAM) as the methyl donor and results in the formation of creatine and s-adenosylhomocysteine (SAH). In rats AGAT is mainly expressed in the kidney and the GAMT is mainly localized in the liver resulting in an inter-organ synthesis of creatine (da Silva et al.2009; Wyss M and Kaddurah-Daouk R. 2000; Edison et al. 2007). The data in human are unclear, and a complete pathway for creatine synthesis may be present in the liver, pancreas and possibly brain. The kidney is estimated to contribute 20–25% of the GAA to total creatine synthesis in humans (Edison et al. 2007). Creatine is eliminated from the body following its spontaneous breakdown to creatinine.

Because of its multi-compartmental and large volume of distribution and a relatively slow rate of turnover, the rate of creatine synthesis has not been quantified systematically in healthy humans. Previous data have relied upon the rate of creatine/creatinine excretion in the urine, organ balance or by using ^{14}C or ^{15}N tracers in a few subjects (Hoberman et al. 1948; Sandberg et al. 1953; Crim et al. 1976).The long duration (days) of the tracer elimination and the errors associated with the measurements of urinary losses has made the study of creatine kinetics in-vivo difficult. We have developed a GC-mass spectrometric method for the simultaneous measurement of tracer enrichment and concentration of GAA and creatine in biological fluids (Kasumov et al. 2009). In the present study, we have applied this technique in order to quantify the rate of creatine synthesis and its pool size in healthy subjects. In addition we have quantified the response to the administration of supplemental creatine and of supplemental amino acids on creatine, glycine and protein kinetics in the same subjects.

Material and methods

Young, healthy subjects (8 males and 7 females) were recruited for the kinetic studies by advertisement. At recruitment, a detailed clinical history was obtained and a physical examination was done. Subjects with a family history of chronic illness were excluded from the study. Other exclusion criteria included intake of nutritional supplements, medication, prior history of creatine supplementation and any known medical condition that may impact skeletal muscle, liver or renal function. All eligible subjects had routine blood chemistries (liver function test, metabolic panel), complete blood count and urinalysis performed prior to the study. The study protocol was reviewed and approved by the institutional review board of the Cleveland Clinic Foundation. Written informed consent was obtained from all participants after fully explaining the procedure. The study was registered with [ClinicalTrials.gov](https://www.clinicaltrials.gov/ct2/show/study/NCT01725503) (NCT01725503).

Anthropometric details of the study subjects are displayed in Table 1. Their blood counts, liver function test and other metabolic parameters were within the normal range (supplement Table 1). Body composition analysis was done by dual-energy-X-ray absorptiometry (DEXA, Lunar iDXA, GE Healthcare). None of the subjects were overweight or obese (mean fat mass ~30% of body weight, Table 1).

Creatine kinetics were measured (i) following 5 days of creatine-free diet (study 1), (ii) following 5 days of supplemental creatine 21 g.d⁻¹ (study 2) and (iii) following 5 days of an amino acid supplement (study 3). Seven subjects (5 males, 2 females) participated in the amino acid supplement study.

Study 1

The study design is displayed in Fig. 1. All subjects were advised a creatine-free (*meat-free*) 75 grams protein daily diet for 5 days prior to the tracer study. Dietary compliance was monitored by the clinical nutritionist. They were instructed not to engage in any exercise for 3 days prior to the tracer infusion study. A 24 hour urine specimen was obtained to measure total creatinine excretion. Subjects reported to the Clinical Research Unit at the Cleveland Clinic at 7 AM, following a 10 hour overnight fast. Their weight, height and vital signs were measured. Two indwelling cannulae were placed in a superficial dorsal vein of each hand, one for tracer infusion and the other to obtain blood samples. The sampling site was kept warm by placing the hand in a thermostat-controlled warm blanket, and kept patent by a constant flow infusion of isotonic saline solution. Accurately weighed amounts of sterile, pyrogen free, [1-¹³C]glycine and L-ring[²H₅]phenylalanine (from Cambridge Isotope Laboratories, Inc., Andover, MA, USA) were dissolved in a sterile 0.45% saline solution in a laminar flow hood. Tracer solutions were further sterilized by filtering through a 0.22 micron filter (Millex; Millipore, Bedford, MA). The tracers were administered as prime-constant rate infusion for 8 hours. Their respective doses were as follows: [1-¹³C]glycine, prime: 10 micromoles.kg.⁻¹, constant infusion: 10 micromoles.kg.⁻¹h⁻¹; L-ring[²H₅]phenylalanine, prime: 2 micromoles.kg.⁻¹, constant infusion: 2 micromoles.kg.⁻¹h⁻¹. The actual rate of infusion was confirmed by gravimetrically measuring the rate of flow using the same tubing and equipment at the end of the study. The concentration of glycine and phenylalanine in the infusate were measured as described in

analytical methods. All tracer solutions were tested in the hospital clinical laboratory and were found to be sterile. A bolus dose (150 mg) of [C^2H_3]creatine monohydrate was administered over 3–5 minutes at the start of the study in order to measure the pool size of creatine. Three subjects, studied initially, received 250 mg bolus creatine. Blood samples in EDTA were obtained at 3 minute intervals for the first 30 minutes, and at 30 minute interval for the duration of the study. Samples of expired air were collected using a Rudolph one-way valve system as described (Kalhan et al. 1998). An aliquot of expired air was transferred into a sampling tube for the measurement of ^{13}C enrichment of the expired CO_2 . The rate of production CO_2 and the rate of consumption of O_2 were measured using an open hood respiratory calorimetry system (Viasys Encore, Cardinal Health, Dublin, OH).

Study 2

All subjects were studied again 2–16 weeks later. They were placed on a regular diet containing a minimum 75gm protein for 5 days prior to tracer study. In addition, they were given supplemental creatine, 21gm/day, 7gm 3 times a day, for five days. Creatine monohydrate for oral supplementation was purchased from General Nutrition Corporation, Pittsburgh, PA. It was dispensed as 7 gram aliquots. Each aliquot was dissolved in orange juice and taken orally. The subjects ingested the creatine without any problem. At the end of five days, the tracer isotope study was performed during fasting as described for study 1.

Study 3

Seven subjects (5 males, 2 females) were studied again while receiving an amino acid supplement. The supplement was given as amino acid capsules, 3 times a day (14.3 grams per day) with meals. Those who could not ingest the capsule were asked to open them and dissolve in juice. Each dose (4.766gms) of the amino acid mixture contained L-leucine 1268.7 mg, L-isoleucine 339.6 mg, L-valine 348.7 mg, L-threonine 295.7 mg, L-lysine 528.4 mg, L-methionine 105.2 mg, L-histidine 52.9 mg, L-phenylalanine 211.4 mg, L-tryptophan 21.2 mg. The amino acid mixture was prepared by Ajinomoto U.S.A., Inc. (Fort Lee, NJ) and packaged into capsule form by Atlantic Essential Products, Inc. (Hauppauge, NY). The tracer study was performed as described in Study 1.

Analytical methods

Enrichment of glycine, serine and phenylalanine were measured using gas chromatography-mass spectrometry (GC-MS). Amino acids in the plasma were separated using mixed-bed ion exchange chromatography as described previously (Kalhan et al. 1998). An *N*-propyl-*n*-acetyl ester derivative of glycine, serine and phenylalanine was prepared according to the method of Adams (Adams, 1974), as described before (Kalhan et al., 1998). The glycine and serine derivatives were separated on a GC-mass spectrometry system (6890N GC System coupled with 5973 mass selective detector; Agilent Technologies, Santa Clara, CA) using HP-INNOWAX capillary column (30m \times 0.25mm \times 0.25 μ m) as described previously (Dasarathy et al. 2009; Kalhan et al. 2011). Positive chemical ionization and selected ion monitoring (SIM) were used to monitor mass-to-charge ratio (*m/z*) for ions 160 and 161 representing unlabeled (m_0) and ^{13}C -labeled (m_1) glycine and *m/z* 232 and 233 corresponding to unlabeled serine and [$1-^{13}C$]serine. For phenylalanine, the ions monitored

were m/z 250 and 255 corresponding to unlabeled and $^2\text{H}_5$ -labeled (m5) phenylalanine. Standards solutions of known enrichments were run simultaneously to correct for variations in instrumentations. The concentration of cysteine, homocysteine and glutathione in the plasma were measured as described previously (Dasarathy et al. 2009). This method measures the total cysteine (cysteine and cysteine) and total homocysteine (free and protein bound) in the plasma.

Calculations

Fat mass and Lean mass were calculated from the body composition study by DEXA, using proprietary software (GE Healthcare). The muscle mass was estimated from the lean mass assuming that muscle mass represents 54% and 48% of total fat free body weight respectively in males and females (Clarys et al. 1984). The rate of appearance of glycine and phenylalanine was calculated using tracer dilution equations during steady state as described previously (Kalhan et al. 1998). The rate of oxidation of glycine was calculated from the rate of CO_2 production and the appearance of ^{13}C in the expired CO_2 using precursor product relationship (Dasarathy et al. 2009).

The pool size of creatine was estimated from the dilution of a given dose of $[\text{C}^2\text{H}_3]$ creatine (m3) in the plasma. As shown in the figure 3, the dilution (enrichment of creatine in the plasma) of the bolus dose creatine tracer revealed a bi-exponential curve suggesting two distinct pools of creatine: a smaller rapidly mixing pool and a second large pool in which a slow distribution of the injected bolus tracer occurred. Using the “curve stripping” process and log transformation of the tracer enrichment time data, the tracer enrichment at time zero, where the best-fit lines cross the y axis, for the two exponents was calculated (Teuscher N. 2011). The estimated dilution of the tracer (m3/m0) at time zero relative to the administered dose was used to estimate the pool size (Q1 and Q2) of creatine in the two compartments.

The fractional rate of synthesis of creatine (FSR) was calculated from the ^{13}C enrichment of the immediate precursor, guanidinoacetic acid, (E_{GAA}) and that of the product, creatine, (E_{C}) following $[1-^{13}\text{C}]$ glycine infusion, as follows: $\text{FSR creatine (\%/day)} = (\text{slope of } E_{\text{C}} / E_{\text{GAA}}) * 1440 * 100$. The absolute rate of creatine synthesis (ASR) equals $\text{FSR} * \text{creatinine pool (Q2)}$.

Statistical Analysis

All data are reported as mean \pm std. dev. Paired t test were used to compare the response to supplemental creatine or with amino acids in the same subject. Correlations and curve fitting for each subject were done using Origin 9 data analysis and graphing software (OriginLab Corporation, Northampton, MA, USA).

Results

The anthropometric characteristics of the study subjects are displayed in Table 1. As shown the study subjects were between 20 and 30 years of age, were in good health and their metabolic laboratory data (supplemental Table 1) were within the normal range. The concentrations of creatinine and urea nitrogen in the plasma also were within the normal range. Their fat mass and lean body mass estimated using DEXA and calculated muscle

mass are displayed. The muscle mass was calculated from the lean body mass using the equations of Clarys et al (1984). The responses to the administration of creatine are described below. Amino acid administration did not have any significant effect on any of the parameters studied (supplemental tables).

Body weight, plasma Creatine, Creatinine, Guanidinoacetic acid and urinary (Table 2)

Creatine supplement did not cause any significant change in the average body weight of the entire group. However when examined separately, in female subjects the increase in body weight (mean ~1.4Kg) was statistically significant ($p=0.002$). Plasma creatine concentration was similar in males and females while on creatine free diet. Five days of creatine supplement caused a marked increase in plasma concentration of creatine (measured ~12 hours after the last creatine dose) in both males and females. The only exception was one male subject who did not show any change in plasma creatine and did not show significant change in other parameters. None the less he is included in all analysis. As reported by others in humans and animals (Derave et al.2004; Edison et al. 2007), there was a significant decrease (50%) in the concentration of guanidinoacetic acid in the plasma in all subjects following creatine supplementation. The plasma concentration of GAA was lower in the female subjects compared with the males on creatine free diet and when receiving creatine supplement (table 2). While on creatine free diet, the plasma concentration of GAA was significantly correlated with estimated muscle mass in all subjects, Pearson $r=0.657$, $P=0.008$.

There was no effect of supplement creatine on plasma creatinine concentration or on the 24h urinary creatinine excretion neither in male nor in female subjects (Table 2). The plasma concentration of creatinine and the 24 hour urinary creatinine excretion were significantly less in the female subjects while on creatine free diet, reflecting their lower muscle mass (Table 2). The plasma concentration of creatinine in the females remained lower than that in the males while on creatine supplement.

Plasma Cysteine, Homocysteine and Glutathione

Creatine supplement caused a significant increase in the plasma concentration of total cysteine in the entire group. Individually the increase was statistically significant only in the females and not in the males. There was no change in the total homocysteine concentration in the plasma following creatine supplement. Total glutathione concentration in the plasma was lower following creatine supplement in the female subjects but not in the males (Table 3). The plasma levels of cysteine, homocysteine and glutathione were not correlated with plasma levels of creatine.

Plasma Amino acid concentration

Five days of creatine supplement resulted in a significant decrease in plasma levels of glycine in all subjects ($p=0.004$). There was a significant increase in plasma levels of citrulline, valine and lysine and a significant decrease in the levels of glutamine and taurine (Table 4). There was no change in the levels of other amino acids in the plasma.

Glycine and Phenylalanine Kinetics

The rates of appearance (Ra) of phenylalanine and glycine were calculated, using tracer dilution equations, at isotopic steady state, achieved in all subjects between 1.5 to 2 hours of tracer infusion. Creatine supplement had no effect on the whole body rate of protein turnover as measured by the rate of appearance of phenylalanine in the plasma neither in male nor in female subjects (Table 5). In contrast there was a significant decrease in the rate of appearance of glycine in the entire group (130.3 +/- 26.9 vs 117.3 +/- 20.9 micromoles/kg/h, $p=0.006$). Glycine Ra decreased in all subjects except two male subjects who showed an increase in Ra following creatine supplement. Although there was a decrease in mean Ra of glycine in both male and female subjects, the decrease was significant only in the female subjects ($p=0.04$). The decrease in glycine Ra in the presence of unchanged Ra phenylalanine suggests a decrease in de-novo synthesis of glycine. Approximately 24% of glycine Ra or ~ 30 micromoles.kg⁻¹.h⁻¹ was oxidized (glycine cleavage system) and $\sim 22\%$ of serine was derived from glycine. The rate of oxidation of glycine decreased significantly in the entire group ($p=0.02$) and in the male subjects ($p=0.02$) following creatine supplement. The fraction of serine derived from glycine was not affected by creatine supplement.

Guanidino acetic acid

Five days of creatine supplement resulted in $\sim 50\%$ reduction in plasma GAA concentration in all subjects (Table 2). As shown in Fig 2 there was a rapid appearance of tracer in the plasma GAA following the prime injection of tracer glycine, suggesting a rapid enrichment of glycine pool at the site/s of synthesis of GAA. This was followed by a slow linear increase in [¹³C] enrichment (m_1) of GAA in the plasma (average slope of m_1 moles % excess in GAA= 0.002/min). Creatine supplement did not impact the slope of enrichment in GAA in either male or female subjects, The average enrichment of GAA between 2nd and the 5th hr was used to calculate the fractional rate of synthesis of creatine. The average enrichment in GAA was 44% and 36% of plasma glycine enrichment respectively on creatine free diet and on creatine supplement (Table 6). The lower GAA/glycine enrichment ratio on creatine supplement diet was due to higher m_1 enrichment of the plasma glycine pool.

Creatine kinetics

As shown above, creatine supplement caused an almost ten folds increase in plasma concentration of creatine in all subjects (Table 2). The kinetics of synthesis of creatine are shown in Table 6. Creatine administration significantly decreased the mean rate of appearance of tracer glycine in the creatine pool (slope) in the entire group ($p=0.005$). However when examined separately by gender, the decrease was statistically significant only in the female subjects. The mean fractional rate of creatine synthesis, calculated based upon the enrichment of the GAA pool, was 27%.day⁻¹ while on creatine free diet and decreased to 18.6%.day⁻¹ following creatine supplement ($p=0.007$). The decrease was statistically significant only in the male subjects.

We estimated the size of the rapidly mixing pools of creatine by examining the kinetics of a bolus dose of [²H₃]creatine. As shown in figure 3 the dilution of the bolus dose of the

tracer revealed bi-exponential kinetics suggesting two different pools or compartments of creatine, a small rapidly mixing pool and a large slowly mixing pool. The estimated sizes of the two pools (Q1 and Q2) are displayed in Table 6. As shown, while on creatine free diet, Q1 was not significantly different in males and females and was approximately 1mmole. In contrast, the second larger pool (Q2) was significantly smaller in the female subjects when compared with the males (mean 19.3 mmoles vs. 36.2 mmoles respectively, $p=0.01$). Creatine supplement caused a five-fold increase in the rapidly mixing pool (Q1) in both males and females and in the entire group while it did not cause a significant change in the larger creatine pool (Q2). Q2 remained significantly smaller in females when compared with the males, as was the case when on creatine free diet. We calculated the rate of synthesis of creatine from the appearance of [^{13}C]creatine (m1) in the larger creatine pool (Q2).

The absolute synthesis rate of creatine was significantly less in the female as compared with the male subjects ($p=0.01$) while on creatine free diet. Creatine supplement caused a significant decrease in the rate of creatine synthesis in the entire group (from 7.41 ± 4.22 mmoles/d to 5.16 ± 2.29 mmoles.d $^{-1}$, $p=0.02$). The decrease in creatine synthesis was statistically significant only in the males.

Amino acid supplement did not have any effect on glycine, phenylalanine or creatine kinetics (Data in supplemental tables).

Discussion

The data from the present study show that creatine supplement for five days, 1) caused a marked increase in plasma concentration of creatine and a decrease in plasma concentration of GAA in all subjects, 2) did not impact plasma creatinine levels or daily urinary excretion of creatinine, 3) caused a five-fold increase in the rapidly mixing creatine pool but had no impact on the larger slowly mixing pool, 4) caused a significant decrease in the rate of de-novo synthesis of glycine while it had no effect on whole body rate of appearance of phenylalanine, 5) caused a significant decrease in the rate of synthesis of creatine. Oral supplementation with amino acids for five days had no measurable effect on any of these parameters. It should be underscored that we examined the response to five days of creatine supplement in the fasting state and that these data cannot be extrapolated to prolonged continuous administration.

Homocysteine, Cysteine and Glutathione

Creatine supplement did not affect the concentration of total homocysteine in the plasma in our study (Table 3). This is in contrast to the previous data in humans where a small but significant decrease was observed in one study (Korzun WJ. 2004) and a non-significant decrease in another (Steenge et al. 2001). In contrast Stead et al observed a 25% decrease in plasma levels of homocysteine in rats maintained on a creatine supplemented diet for two weeks (Stead LM et al. 2001). The decrease in plasma homocysteine concentration has been attributed to a decrease in the methylation demands and therefore decreases in transmethylation of methionine, as a result of creatine supplement (Stead LM et al. 2001). The lack of effect on plasma homocysteine in our study may be related to the short duration of creatine supplement, to a difference in the dose of creatine given; although the use of

creatine free diet in our study 1 by increasing the methylation demand should have made the effect of creatine supplement more evident. In the study by Korzun (2004) and by Steenge et al. (2001), creatine supplement was given respectively for 4 and 8 weeks. Additionally since the decrease in plasma homocysteine has been attributed to decrease in methylation demand the decrease in creatine synthesis (20–40%), in our study, may not have been enough to show any significant differences in plasma homocysteine. The physiological reason for the increase in plasma cysteine and glutathione following creatine supplement cannot be discerned from these data and may be related to changes in glutathione metabolism.

Amino acids

There was a significant decrease in plasma concentration of glycine in all subjects following creatine supplement, likely related to decreased demands for glycine for creatine synthesis (discussed below). The changes in plasma taurine, glutamine and other amino acids may be the consequence of subtle and not measurable changes in the metabolism of the skeletal muscle or the result of changes in tissue osmolarity as a result of retention of water (Powers et al. 2003).

Creatine supplement had no effect on the whole body rate of protein turnover as measured by the rate of appearance of phenylalanine. These data are consistent with the previous studies in literature which did not show any significant effect of creatine supplement on protein metabolism in the skeletal muscle in-vivo (Louis et al 2003; Parise et al 2001; Ingwall et al. 2000); although there was significant increase in the skeletal muscle creatine levels. Parise et al (2001) did observe a decrease in whole body rate of appearance of leucine and its rate of oxidation, in response to creatine in men only and not in women. They attributed it to possible change in hepatic metabolism of leucine.

In contrast to no change in phenylalanine Ra, there was a significant decrease in the rate of appearance of glycine in the plasma (Table 5). The rate of decarboxylation of glycine (glycine cleavage system) also decreased following supplemental creatine although the fraction of serine derived from glycine was not changed. The lower Ra of glycine in the presence of unchanged whole body rate of proteolysis suggests a lower rate of de-novo synthesis of glycine. Although the exact mechanism of the decrease in the rate of synthesis of glycine cannot be discerned, it is likely to be related to the decrease in the requirement of glycine for creatine synthesis and to a decrease in methylation demand i.e. decrease in the need for the methyl group as a result of lower rate of creatine synthesis (Stead LM et al. 2001). As discussed by Melendez-Hevia and colleagues (2008) and by Hofmeyr and Cornish-Bowden (2000), the stoichiometry of glycine biosynthesis from serine via glycine hydroxyl methyl transferase and its disposal via consumption of C1 units transferred via THF, results in unique metabolic constraints so that the rate glycine biosynthesis cannot exceed the flux of consumption of the methyl groups via THF or that they are closely linked. Based upon this analysis, we attribute the lower rate of glycine synthesis following creatine supplement to the decrease in the demand for glycine and for methyl groups for creatine synthesis.

Guanidinoacetic acid

As reported by Derave et al (2004) in healthy humans and by Edison and colleagues (2007) in rat and humans, oral creatine supplement, in the present study, caused a marked (50%) decrease in the plasma concentration of GAA (Table 2). The decrease in plasma GAA has been attributed to decreased synthesis of GAA. We did not see a change in tracer enrichment of GAA following creatine supplement (Table 6) possibly because of a small size of the GAA pool and its rapid equilibrium and enrichment following tracer priming of the glycine pool. Since the glycine pool in the tissues is the only source of GAA and because of rapid equilibrium between glycine and GAA, a change in rate of synthesis of GAA will not be reflected in the tracer enrichment of plasma GAA. This was the case in our study (Fig. 2 and Table 6). The decrease in the synthesis of GAA has been attributed to pre-translation inhibition and to lower activity of AGAT in the kidney caused by supplemental creatine (McGuire et al. 1984). In the rat, most of the GAA is produced in the kidney (Edison et al. 2007). Creatine supplement in the rat suppressed the synthesis of GAA in the kidney by decreasing both the amount of protein and the activity of AGAT (Edison et al. 2007). There was a close correlation between the rate of production of GAA by the kidney and urinary rate of excretion of creatinine further providing evidence for the kidney to be the major site of GAA synthesis in the rat (Edison et al. 2007). These data have been interpreted to suggest that at least in the rat there is an inter-organ synthesis of creatine, whereby GAA is synthesized in the kidney, released into circulation and converted to creatine in the liver. In contrast in humans, the renal production of GAA amounted to only 11–12% of creatinine excretion by the kidney. The authors interpreted these data to suggest that in humans, GAA is possibly produced at sites other than the kidney (Edison et al. 2007). Such an inference is also suggested by the presence of AGAT in organs other than the kidney (Wyss, M. and Kaddurah-Daouk, R. 2000).

The slow linear increase in ^{13}C enrichment (m1) of the GAA in the plasma (average slope moles % excess = 0.002/min) suggests multiple heterogeneous pools of GAA in different organs and tissues of varying size and kinetics as well as the differences in intracellular glycine flux and pools in different organs (Arend et al. 1995; Garfinkel D and Lajtha A. 1963)). These data are consistent with the study of Edison et al. (2007) who showed that unlike in rats, in humans only a small proportion of whole body GAA is synthesized in the kidney and released into the circulation.

Creatine

Plasma creatine concentration increased almost ten fold following supplemental creatine. The creatine levels in the plasma were measured in the fasting state, ten to twelve hours after the last creatine dose. These data are similar to those reported by Brault and colleagues (2007). Corresponding to increase in plasma creatine, there was a fivefold increase in the rapidly mixing creatine pool (Q1) in all subjects (Table 6). The estimated volume of distribution of Q1 was approx. 35% of the body weight suggesting distribution in little more than extracellular compartment. Interestingly, the larger slow mixing pool (Q2) did not change following creatine supplement. This may be related to mixing with the intracellular pools of creatine. The calculated size of the creatine pools is likely an under-estimation because of loss/excretion of some of the bolus tracer creatine in the urine prior to mixing in

the entire pool. This is particularly true following creatine supplementation (Harris et al. 1992; Burke et al. 2001; Rawson et al. 2004) when the concentration of creatine in the plasma was high. Previous data in the literature show that as much as 30 to 40% of the creatine supplement is excreted in the urine (Harris et al. 1992; Burke et al. 2001). We could not correct for this loss because we had not obtained timed urine collections. In addition the multi-compartmental distribution of creatine and the regulation of its uptake by its transporter create some inherent problems for the estimation of pool size of creatine (Elloot et al. 2005; Snow et al. 2001). Although the plasma concentration of creatine increased markedly in all subjects, the larger creatine pool (Q2) increased, not significant, only in the females, a group with the smaller initial creatine pool compared with the males. Interestingly, the female subjects also showed a significant increase in body weight following creatine supplement, likely related to the retention of water in the body (Powers et al. 2003). The absolute rate of synthesis of creatine in female subjects, while on creatine free diet, was ~50% of that of male subjects (Table 6). The estimated muscle mass of the female subjects was also ~50% of that of males (Table 1). Even though the fractional rate of synthesis of creatine decreased in all subjects, the absolute rate of synthesis decreased in males only. This was related to unchanged creatine pool in males and an increase in the creatine pool in females.

Our estimates of the rate of synthesis of creatine are the first detailed analysis in young healthy men and women. The measured rates of creatine synthesis for men (1.3gms.d^{-1}), while on creatine free diet although comparable with those in literature, are less than those reported (1.6gms.day^{-1}). We could not find similar measurements for women. Previous data on a small group of subjects, mostly men, have used the rate of excretion of creatinine and creatine in the urine, organ balance data, or ^{15}N , ^{13}C or ^{14}C labeled tracers of creatine to estimate the rate of creatine synthesis (Cockcroft and Gault, 1976; Sandberg et al. 1953; Hoberman et al. 1948; Crim et al. 1976; Kreisberg et al. 1970). Cockcroft and Gault (1976) measured the rate of creatinine excretion in 22 male subjects age 18 to 29 years to be $\sim 24\text{mg.kg}^{-1}.\text{24h}^{-1}$ corresponding to $\sim 1.6\text{gms.d}^{-1}$ for a 70 kg person. These measurements could be overestimates since urinary creatine excretion has been reported to be directly related to dietary creatine intake and these subjects were not on creatine free diet. The studies using organ balance data and those using tracer labeled creatine were done on a small group of men, one to four subjects, and gave variable results (Hoberman et al. 1948; Crim et al. 1976). In contrast to the studies in literature, we studied both men and women over a short duration and observed less variability than that seen in previous tracer studies. The short duration of the study and ease of analysis allows for examination of effects of other nutritional and metabolic interventions. In addition, we studied subjects only in the fasting state, while the reported data in literature includes both fasting and fed state. The effect of feeding and dietary nutrient intake on creatine synthesis has not been explored in detail.

Our data confirm the estimates by Brosnan and colleagues, regarding metabolic burden of creatine synthesis on labile methyl groups provided by S-adenosylmethionine (Brosnan et al. 2011). Based upon measured transmethylation flux of 16.3mmoles per day for a 70kg person (MacCoss et al. 2001), our data suggest that creatine synthesis consumes approximately

45% of the S-adenosylmethionine generated in the transmethylation flux of methionine. These estimate is similar to the 40% calculated by Brosnan et al (2011).

Potential weaknesses: Since we did not supply the food when the subjects were on creatine free diet, it is possible that they may not have adhered to the diet rigorously. This would result in an underestimation of rate of creatine synthesis. Even though we did monitor for dietary compliance by frequent contacts, one male subject appeared to have not taken creatine since his plasma creatine concentration remained unchanged following supplement creatine. We did include his data in the reported analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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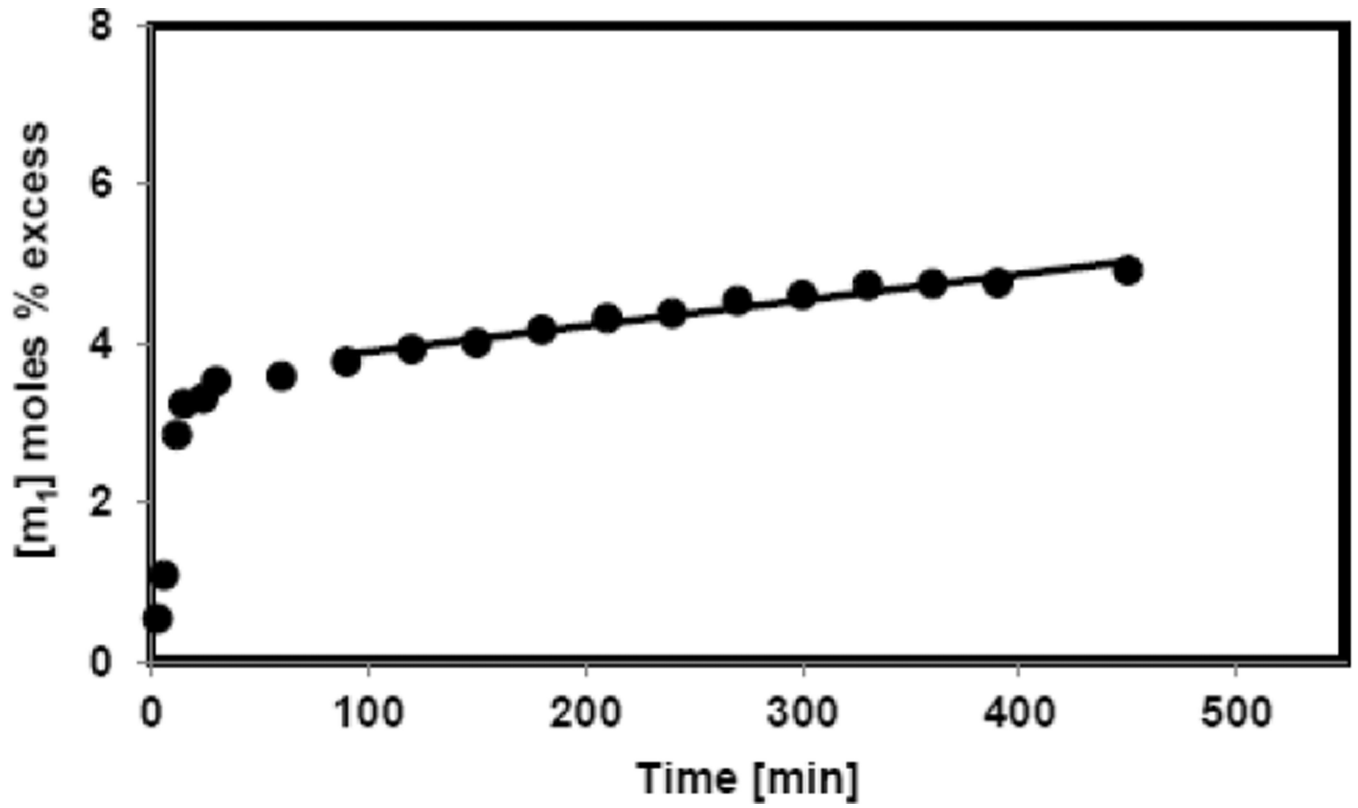


Fig. 2.
The time course of appearance of [1-¹³C]glycine (m₁) in the plasma guanidinoacetic acid following prime constant rate infusion of tracer glycine in one subject. (Details in the text).

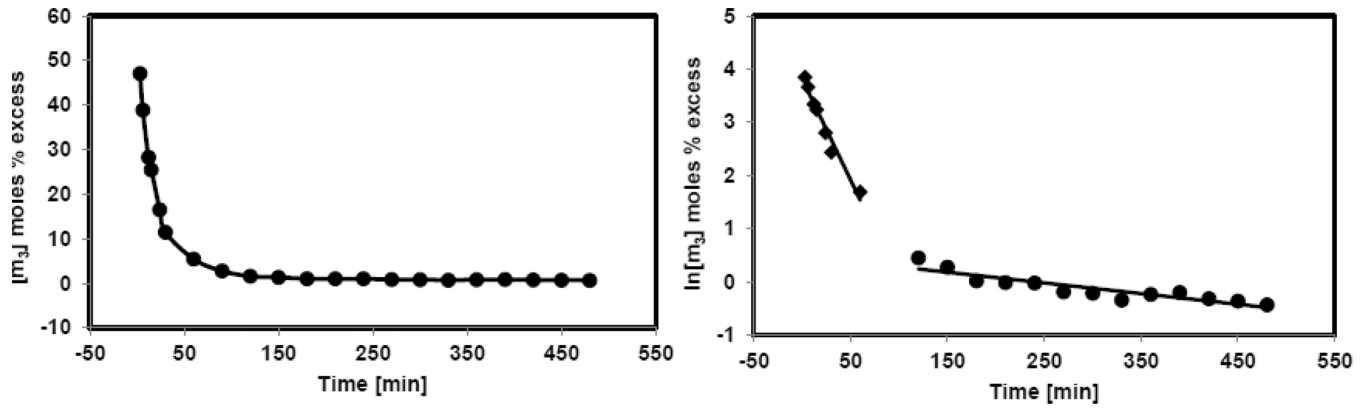


Fig. 3. Time course of dilution of the bolus $[C^2H_3]$ creatine in plasma of one subject is shown. Curve stripping was employed to calculate the size of the creatine pools.

Table 1

Characteristics of the Study Population

Gender	Age years	Weight kg	BMI kg/m ²	Fat Mass [§] kg	Fat free Mass [§] kg	Muscle Mass kg	Hgb g/dL
Males (8)	25.6 ± 4.7	89.9 ± 18.8 (67.9 – 117.8)*	27.9 ± 5.9	25.2 ± 13.5	64.1 ± 8.1	34.6 ± 4.4	15.0 ± 1.0
Females (7)	22.1 ± 2.3	59.1 ± 11.8 (48.9 – 79.2)	23.3 ± 4.6	18.1 ± 7.3	40.6 ± 4.0	19.8 ± 1.6	12.5 ± 1.0
All	24.0 ± 4.1	75.6 ± 21.9	25.7 ± 5.7	21.9 ± 11.2	53.1 ± 13.7	27.7 ± 8.3	13.8 ± 1.6

All data are mean ± SD

* (range)

§ Estimated by Dual-energy X-ray Absorptiometry (DEXA)

Hgb: blood hemoglobin.

Table 2

Effect of Creatine Supplementation on Body Weight, Plasma Levels of Creatine, Guanidinoacetic Acid, Creatinine and 24 Hour Creatinine Excretion

<u>Creatinine</u>	<u>Weight</u> kg	<u>Creatine</u> micromoles/L	<u>GAA</u> micromoles/L	<u>Plasma</u> mg/dL	<u>24hr creatinine (urine)</u> gram
<u>Creatine-free</u>					
Males (8)	89.9 ± 18.8	39.16 ± 10.14	3.12 ± 0.66	0.93 ± 0.21	2.07 ± 0.59
Females (7)	59.1 ± 11.8	37.92 ± 8.73	2.02 ± 0.54 [§]	0.74 ± 0.12 [*]	1.04 ± 0.17 ^{§§}
All (15)	75.6 ± 21.9	38.58 ± 9.19	2.61 ± 0.82	0.84 ± 0.19	1.59 ± 0.68
<u>Creatine Supplement</u>					
Males (8)	90.4 ± 18.8	302.22 ± 198.61 [†]	1.66 ± 0.88 [*]	0.94 ± 0.16	2.40 ± 0.60
Females (7)	60.5 ± 11.2 [*]	340.34 ± 89.05 ^{††}	0.89 ± 0.25 ^{*•}	0.68 ± 0.09 ^{••}	1.26 ± 0.38
All (15)	76.5 ± 21.7	320.01 ± 153.32 ^{**}	1.30 ± 0.76 ^{**}	0.82 ± 0.18	1.87 ± 0.77

GAA: Guanidinoacetic acid

Compared with creatine free study,

p = †0.008;

* 0.0002;

†† 0.0001;

** 0.00

Compared with males,

p = §0.004;

§§ 0.001;

• 0.05;

•• 0.002

Table 3

Plasma Homocysteine, Cysteine and Glutathione

	<u>Cysteine</u>	<u>Homocysteine</u>	<u>Glutathione</u>
<u>Creatine-free</u>			
Males (8)	255.2 ± 23.3	9.2 ± 1.6	5.6 ± 0.7
Females (7)	227.8 ± 35.2	7.4 ± 1.3	5.6 ± 2.5
All	242.4 ± 31.7	8.4 ± 1.7	5.6 ± 1.7
<u>Creatine Supplement</u>			
Males (8)	268.7 ± 35.8	9.7 ± 2.3	5.0 ± 0.7 [†]
Females (7)	258.3 ± 34.2 [*]	8.4 ± 0.9	4.4 ± 0.9
All	263.9 ± 34.2 ^{**}	9.1 ± 1.8	4.7 ± 0.8

Data are micromoles/L; mean ± SD

Compared with creatine-free,

p = *0.01;

** 0.004;

[†]p=0.05

Table 4

Plasma Amino Acids

	Study 1 Creatine-free diet	Study 2 Creatine-supplement
ASP A	9.0 ± 1.8	8.0 ± 2.3
GLU A	36.9 ± 11.7	37.7 ± 11.9
ASN	45.4 ± 7.2	48.2 ± 6.8
SER	114.0 ± 30.9	111.3 ± 21.7
GLN	582.9 ± 62.2	525.6 ± 43.5*
HIST	84.0 ± 8.5	86.6 ± 11.2
GLY	271.7 ± 52.7	241.5 ± 32.9**
CIT	28.8 ± 5.6	31.4 ± 4.8 [†]
THR	134.4 ± 29.9	145.5 ± 21.9
ALA	289.1 ± 96.3	295.1 ± 72.7
ARG	104.4 ± 20.4	115.3 ± 22.5
TAUR	35.6 ± 4.8	32.1 ± 4.9 ^{††}
TYR	59.1 ± 14.7	60.7 ± 12.3
ABA	24.9 ± 4.9	27.2 ± 4.5
METH	22.6 ± 3.3	23.6 ± 2.6
VAL	211.6 ± 45.7	230.2 ± 38.2 [†]
TRY	50.0 ± 7.4	51.7 ± 7.6
PHE	58.5 ± 6.2	57.2 ± 5.6
ISO LEU	55.8 ± 12.4	56.5 ± 12.4
LEU	116.5 ± 24.0	119.8 ± 22.9
ORN	61.9 ± 11.3	63.0 ± 10.9
LYS	204.4 ± 50.2	235.2 ± 41.5 [†]

P = *0.01;

** 0.004;

[†] 0.02;^{††} 0.0005

Data are micromoles/L; mean ± SD

Table 5

Phenylalanine and Glycine Kinetics

	Phenylalanine Ra	Glycine Ra	Serine from Glycine	VCO₂	Glycine oxidation
	$\mu\text{moles.kg}^{-1}.\text{h}^{-1}$	$\mu\text{moles.kg}^{-1}.\text{h}^{-1}$	%	$\text{L.kg}^{-1}.\text{h}^{-1}$	$\mu\text{moles.kg}^{-1}.\text{h}^{-1}$
<u>Creatine-free</u>					
Males (8)	28.2 ± 5.7	125.5 ± 32.7	21.9 ± 2.3	0.13 ± 0.02	30.9 ± 5.9
Females (7)	29.0 ± 6.0	135.8 ± 19.2	24.3 ± 4.2	0.15 ± 0.01	36.1 ± 4.1
All	28.6 ± 5.7	130.3 ± 26.9	23.0 ± 3.4	0.14 ± 0.02	33.2 ± 5.7
<u>Creatine Supplement</u>					
Males (8)	27.2 ± 4.7	114.7 ± 21.2	20.8 ± 1.4	0.12 ± 0.02	27.2 ± 6.3 [§]
Females (7)	28.4 ± 6.1	120.1 ± 21.9 [*]	22.7 ± 1.1	0.14 ± 0.03	31.8 ± 6.5
All	27.7 ± 5.2	117.3 ± 20.9 ^{**}	21.7 ± 1.6	0.13 ± 0.02	29.2 ± 6.6 [§]

Mean ± SD

Significantly different from creatine-free,

p = *0.04;

** 0.006,

§ p=0.02

Table 6

 ^{13}C Enrichment of GAA, Creatine Pool and Kinetics of Creatine Synthesis

	m_1 Enrichment in GAA (moles % excess)		Creatine Pool mmoles		Creatine Synthesis		
	Q1	Q2	Slope	Fraction % day ⁻¹	mmoles day ⁻¹	Gms day ⁻¹	
<u>Creatine-free</u>							
Males (8)	3.09 ± 0.60	1.2 ± 0.57	36.2 ± 13.51	0.00059 ± 0.0003	26.98 ± 10.16	9.64 ± 4.48	1.26 ± 0.59
Females (7)	2.97 ± 0.43	0.8 ± 0.32	19.3 ± 5.46*	0.00055 ± 0.0003	27.03 ± 13.29	4.85 ± 1.97	0.64 ± 0.26
All (15)	3.03 ± 0.51	1.0 ± 0.49	28.3 ± 13.41	0.00057 ± 0.0003	27.00 ± 11.28	7.41 ± 4.22	0.97 ± 0.55
<u>Creatine Supplement</u>							
Males (8)	3.00 ± 0.63	5.8 ± 3.56 [§]	31.3 ± 6.03	0.00041 ± 0.0002	19.75 ± 6.95 [†]	6.25 ± 2.44 [†]	0.82 ± 0.32 [†]
Females (7)	2.60 ± 0.60	5.2 ± 1.23 ^{§§}	22.6 ± 5.74*	0.00031 ± 0.0001 [†]	17.36 ± 3.73	3.91 ± 1.37	0.51 ± 0.18
All (15)	2.81 ± 0.63	5.5 ± 2.65 ^{§§}	27.3 ± 7.25	0.00036 ± 0.0001 ^a	18.63 ± 5.63 ^b	5.16 ± 2.29 ^{††}	0.68 ± 0.30 ^{††}

Significantly different compared with males

* p = 0.01

Significantly different compared with creatine free study, paired analysis,

p = §0.01;

§§0.00;

† 0.04;

†† 0.02;

^a 0.005;^b 0.007