Amino Acids (1998) 14:371–377

Amino
Acids
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Printed in Austria

Renal excretion of γ-carboxyglutamic acid and metabolic rate in 3–18 years old humans

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Accepted December 16, 1997

Summary. The modified amino acid γ-carboxyglutamic acid (Gla) occurs in several proteins such as prothrombin, blood coagulation factors VII, IX and X, proteins C, S and Z as well as matrix Gla protein and osteocalcin. The amount of Gla excreted in urine is a common indicator of the whole-body degradation of these proteins. We have determined the renal excretion rates of Gla in 3, 6, 10, 14 and 18 years old male and female human subjects (n = 14 per age group and sex) and calculated the respective resting metabolic rates (RMR) on the basis of the body weights using published formulas. We found high correlations between the excretion rates of Gla (μmol/d/kg body weight) and the RMR (kJ/d/kg body weight) in the females (n = 70) of r = 0.70 (y = 0.003x + 0.29) and in the males (n = 70) of r = 0.70 (y = 0.0038x + 0.27) and in all subjects (n = 140) of r = 0.69 (y = 0.0035x + 0.27); $p < 0.01$. We postulate that in children and adolescents a causal relationship exists between the whole-body degradation rate of Gla containing proteins and the metabolic rate.

Keywords: Amino acids – γ-Carboxyglutamic acid – Metabolic rate – Urinary protein catabolites – Reactive oxygen species

Introduction

γ-Carboxyglutamic acid (Gla) is formed post-translationally by vitamin-K-dependent carboxylation of specific glutamic acid residues in proteins involved in hemostasis or bone metabolism namely prothrombin, blood coagulation factors VII, IX and X, proteins C, S and Z or matrix Gla protein and osteocalcin (Vermeer, 1990; Vermeer et al., 1995; Ferland et al., 1993). As the result of the turnover of the Gla-containing proteins Gla is liberated and quantitatively excreted in urine as has been demonstrated in rats (Shah et al., 1978). Therefore, the amount of urinary excreted Gla over time is a common indicator of the whole-body degradation rate of the Gla-containing proteins.

In different mammalian species of various body weights there is a high positive correlation between resting metabolic rates (RMR) and whole-body protein turnover rates determined by isotopic methods (Waterlow, 1984). We have found in different mammalian species of various body weights that the degradation rates of skeletal muscle protein as well as the renal excretion rates of Gla are also positively correlated with the RMR (Schöch and Topp, 1994). From these findings we have hypothesized that there is a causal relationship between the body-size-related metabolic rate and the degradation rates of proteins. It is well known that reactive oxygen species (ROS) can be involved in degradation of proteins (Grune et al., 1995). It is tempting to speculate that ROS are generated dependent on the consumed oxygen which could attack proteins and initiate their degradation.

In the present study we have determined in 3, 6, 10, 14 and 18 years old male and female children and adolescents the correlations between the renal excretion rates of Gla (μmol/d/kg body weight) and the RMR (kJ/d/kg body weight). The RMR of the subjects were calculated on the basis of their body weights using published empirical formulas (Schofield, 1985).

Material and methods

Subjects and urine collection

Fourteen female and fourteen male healthy subjects in each age group 3, 6, 10, 14 and 18 years were enrolled in the study. All subjects were participants of the Dortmund Nutritional and Anthropometric Longitudinally Designed Study (DONALD), in which the normal developement of 3 to 18 years old children and adolescents is investigated. The survey was approved by the international Scientific Commission of the Forschungsinstitut für Kinderernährung. Food intake was ad libitum. One pool-urine per subject was collected quantitatively with controlled collection time ($\bar{x} \pm$ SD: 1373 $\pm$ 154 min.). The pooled urine was frozen at -20°C until analysis. The determined amounts of Gla in the pooled urine samples were extrapolated to 24 h values.

HPLC-determination of Gla in urine

The frozen urine samples were thawed for 20 min. at 37°C. To 0.3 ml urine 0.3 ml 4 M KOH was added and the sample was hydrolyzed for 20 hrs. at 95°C. Thereafter, the sample was cooled to room temperature and completely transferred into a centrifuge vial along with 2 washings of 600 μl H_2O. Then 0.1 ml saturated $KHCO_3$ was added and the sample was neutralized to pH 7 with concentrated $HClO_4$. The sample was cooled on ice for 30 min. and the precipitate was centrifuged at 4°C and 1,100 g for 10 min. A 40 μl aliquot of the supernatant was used for the HPLC-determination of Gla. To this 40 μl sample 40 μl o-phthaldialdehyde (OPA) reaction solution was added using an autosampler. The original OPA solution consisted of 50 mg OPA (Fluka), 4 ml methanol (Merck), 0.5 ml 1 M potassium borate buffer, pH 10.4 (Pierce), 0.05 ml 2-mercaptoethanol (Serva). To 500 μl of this original OPA solution 1,000 μl of the borate buffer was added before use. The urine sample and OPA-reaction solution was mixed by the autosampler and left standing for exactly 3.5 min. at 9°C. Then a 40 μl aliquot was injected onto a precolumn (40 $\times$ 4.6 mm) connected to a maincolumn (250 $\times$ 4.6 mm) filled with Nucleosil 120-5 C_{18} (Macherey-Nagel). The temperature of the columns was held constant at 37°C. The following binary gradient of eluent A (50 mM Na-acetate in 0.5% acetonitril, pH 7.2) and eluent B (composed of 40% eluent A and 60% acetonitril) was run at 0.8 ml/

min.: 0–6 min. 100% A, 7–18 min. linear to 90% A and 10% B, 19–35 min. linear to 50% A and 50% B, 36–50 min. 50% A and 50% B, 51–58 min. linear to 100% A. The OPA-Gla-derivative was quantified by fluorescence detection ($\lambda_{excitation}$ 340 nm, $\lambda_{emission}$ 455 nm). For reference a 4 mM standard solution of Gla (Fluka) was used.

Calculation of the RMR

The RMR was calculated using the following formulas after Schofield (1985):

For 3 and 6 years old females: RMR (kJ/d/kg wt) = (0.085 wt + 2.033)*1,000/kg wt;
for 10, 14 and 18 years old females: RMR (kJ/d/kg wt) = (0.056 wt + 2.898)*1,000/kg wt;
for 3 and 6 years old males: RMR (kJ/d/kg wt) = (0.095 wt + 2.110)*1,000/kg wt;
for 10, 14 and 18 years old males: RMR (kJ/d/kg wt) = (0.074 wt + 2.754)*1,000/kg wt.

Results

Figure 1 shows a typical chromatogram of a human urine sample after precolumn derivatization with OPA. The Gla-OPA derivative elutes after 5 min.

In Table 1 the average body weights, RMR and Gla excretion rates per kg wt are given for 3, 6, 10, 14 and 18 years old female and male subjects. The RMR and the excretion rates of Gla decrease with age in both sexes. The RMR is on average approx. 2.1 times higher in the 3 years olds than the 18

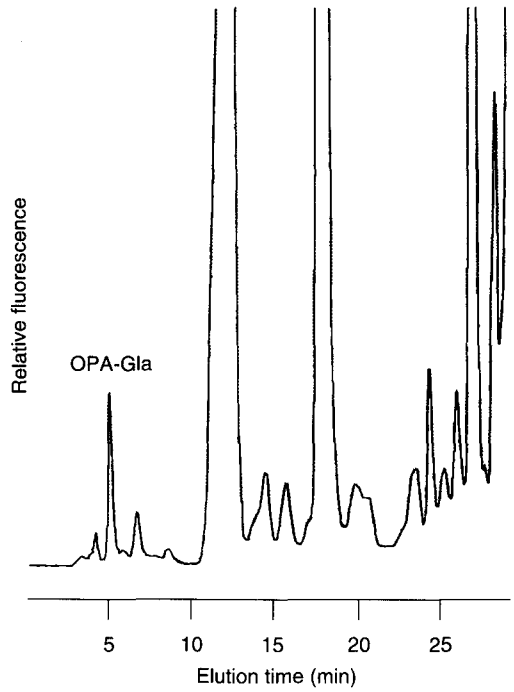

Fig. 1. HPLC separation of γ-carboxyglutamic acid (*Gla*) from a human urine sample after precolumn derivatization with o-phthaldialdehyde (*OPA*). The peak corresponds to 48 pmol Gla

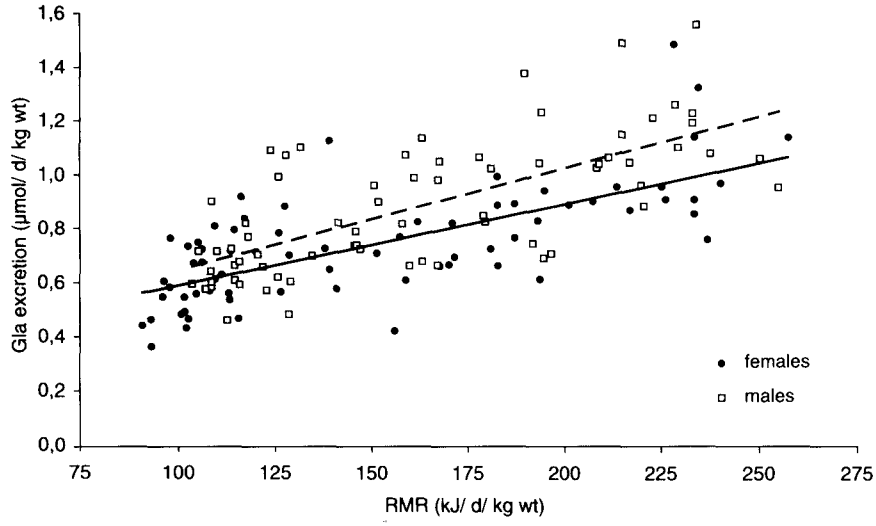

Fig. 2. Correlations between resting metabolic rates (*RMR*) and renal excretion rates of Gla in 3, 6, 10, 14 and 18 years old female subjects ($n = 70$, $r = 0.70$, $p < 0.01$, —— $y = 0.003x + 0.29$) and male subjects ($n = 70$, $r = 0.70$, $p < 0.01$, ------ $y = 0.0038x + 0.27$); in all subjects: $r = 0.69$, $y = 0.0035x + 0.27$

years old subjects. The average excretion rates of Gla per kg wt differ between these two age groups by a similar factor of approx. 1.8. However, even though the average RMR decline continuously from the 3 to the 18 years old subjects the average excretion rates of Gla do not decrease in the 14 years olds (cf. discussion).

As shown in Fig. 2 the excretion rates of Gla and the RMR per kg body weight are highly correlated in female and male subjects.

Discussion

In our study we demonstrate that the renal excretion rates of Gla per kg body weight are significantly correlated with the RMR per kg body weight in healthy 3, 6, 10, 14 and 18 years old female and male subjects.

Urinary Gla originates from the degradation of proteins involved in bone metabolism and hemostasis. It has been suggested that age dependent changes in urinary Gla excretion may be related to bone formation assuming that urinary Gla mainly originates from the turnover of osteocalcin (Gundberg et al., 1983; Goto et al., 1994). However, in these studies urinary Gla was related to urinary creatinine. The excreted amount of creatinine over time is dependent on skeletal muscle mass which per kg body weight is lower in young children than older subjects (Plenert and Heine, 1978; Wang et al., 1996). Therefore, the higher Gla/creatinine quotient in the young children is partly due to the lower muscle mass. On the other hand it has been estimated by others that only about 10–20% of Gla in urine originates from the turnover

Table 1. Average body weights, resting metabolic rates (RMR) and renal excretion rates of γ-carboxyglutamic acid (Gla) in 3, 6, 10, 14 and 18 years old female and male subjects

Age (years)	Sex[a]	Body weight (kg)	RMR[b] (kJ/d/kg wt)	Gla-excretion (μmol/d/kg wt)
3	f	14.5 ± 1.7	227.1 ± 15.6	1.00 ± 0.20
6	f	21.7 ± 3.0	180.2 ± 12.6	0.78 ± 0.13
10	f	37.6 ± 8.6	136.8 ± 17.9	0.70 ± 0.18
14	f	54.6 ± 8.1	110.1 ± 8.0	0.70 ± 0.13
18	f	66.3 ± 9.5	100.6 ± 6.4	0.55 ± 0.11
3	m	15.8 ± 1.4	229.3 ± 12.3	1.16 ± 0.19
6	m	22.9 ± 4.0	189.6 ± 14.8	1.00 ± 0.19
10	m	34.4 ± 5.9	156.0 ± 12.2	0.81 ± 0.15
14	m	53.8 ± 10.5	127.1 ± 10.3	0.81 ± 0.20
18	m	74.9 ± 9.6	111.3 ± 4.7	0.65 ± 0.10

[a] *f* Female, *m* male; [b] Formulas for calculating RMR are given under Materials and methods.

of osteocalcin, whereas the main part stems from the turnover of proteins involved in blood coagulation (Lian et al., 1988; Hauschka et al., 1989; Ferland et al., 1993).

About 20% of the synthesized osteocalcin is not bound to the hydroxyapatite matrix in bone but is set free in the bloodstream and the serum level of osteocalcin is regarded as a marker of bone formation (Cole et al., 1985; Hauschka et al., 1989; Vermeer et al., 1995). However, in a recent longitudinal study of children during their first 9 months of life no correlation was found between serum osteocalcin levels and linear growth velocity (Fleischer et al., 1992). Using the above estimation from Lian et al. (1988) and Hauschka et al. (1989) that 10–20% of Gla in urine stems from osteocalcin turnover and assuming that the factor 1.8 difference in the Gla excretion between the 3 and 18 years old subjects in our study (Table 1) is caused by a higher bone formation this would result in a 4–8 fold higher osteocalcin turnover in the 3 years olds than the 18 years old subjects. However, values from literature of serum osteocalcin levels in male 3 years old subjects are in the same range or lower than the corresponding values in 18 years old subjects (Cole et al., 1985; Hauschka et al., 1989). On the other hand a pronounced increase in serum osteocalcin in 10 and 14 years old subjects indicating an elevated turnover of osteocalcin has been described by the same authors. The latter may contribute to the halted decrease in the Gla excretion of the 14 years old subjects observed in our study (Table 1).

Altogether we conclude from the significant correlation between the excretion rates of Gla and the RMR in our study (Fig. 2) that the degradation rates of all Gla containing proteins (involved in hemostasis and bone metabolism) are correlated with the RMR and that there is a causal relationship between the degradation rates of the Gla containing proteins and the metabolic rate. The results of the present study are in accordance with our previous

findings in different mammalian species of various body weights which also reveal high correlations between the RMR and the excretion rates of Gla as well as the turnover of skeletal muscle protein estimated by the renal excretion of 3-MeHis (Schöch and Topp, 1994). Furthermore, we have found in differently sized mammals that the whole-body degradation rates of tRNA, rRNA and mRNA are also highly correlated with the RMR (Schöch and Topp, 1994). The correlation between the whole-body degradation of special proteins and the metabolic rate could be a general phenomenon due to the fact that in differently sized mammalian species the whole-body protein turnover is also highly correlated with the metabolic rate (Waterlow, 1984). This could mean that the half life of proteins is inter alia an inverse function of the metabolic rate. Whether this is due to oxidation of proteins by reactive oxygen species produced dependent on the oxygen consumed remains a matter for speculation at the moment.

Acknowledgements

This work has been funded by the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen and by the Bundesministerium für Gesundheit.

References

Cole DEC, Carpenter TO, Gundberg CM (1985) Serum osteocalcin concentrations in children with metabolic bone disease. J Pediatr 106: 770–776

Ferland G, Sadowski JA, O'Brien ME (1993) Dietary induced subclinical vitamin K deficiency in normal human subjects. J Clin Invest 91: 1761–1768

Fleischer Michaelsen K, Sidenius Johansen J, Samuelson G, Price PA, Christiansen C (1992) Serum bone γ-carboxyglutamic acid protein in a longitudinal study of infants: lower values in formula-fed infants. Pediatr Res 31: 401–405

Goto K, Kato S, Sumiya S, Uehara S, Hirayama A, Sakamoto W, Tokumitsu Y, Takada M (1994) Urinary levels of γ-carboxyglutamic acid and its clinical significance. Biol Pharm Bull 17: 142–145

Grune T, Reinheckel T, Joshi M, Davies KJA (1995) Proteolysis in cultured liver epithelial cells during oxidative stress. J Biol Chem 270: 2344–2351

Gundberg CM, Lian JB, Gallop PM (1983) Measurements of γ-carboxyglutamate and circulating osteocalcin in normal children and adults. Clin Chim Acta 128: 1–8

Hauschka PV, Lian JB, Cole DEC, Gundberg CM (1989) Osteocalcin and matrix Gla protein: vitamin K-dependent proteins in bone. Physiolog Rev 69: 990–1047

Lian JB, Gundberg CM (1988) Osteocalcin: biochemical considerations and clinical applications. Clin Orthop Relat Res 226: 267–291

Plenert W, Heine W (1984) Normalwerte: Untersuchungsergebnisse beim gesunden Menschen unter besonderer Berücksichtigung des Kindesalters. Karger, Basel

Shah DV, Tews JK, Harper AE, Suttie JW (1978) Metabolism and transport of γ-carboxyglutamic acid. Biochim Biophys Acta 539: 209–217

Schofield WN (1985) Predicting basal metabolic rate, new standards and review of previous work. Hum Nutr: Clin Nutr 39C [Suppl] 1: 5–41

Schöch G, Topp H (1994) Interrelations between the degradation rates of RNA and protein and the energy turnover rates. In: Räihä NCR (ed) Protein metabolism during infancy. Raven Press, New York, pp 49–52

Vermeer C (1990) γ-Carboxyglutamate-containing proteins and the vitamin K-dependent carboxylase. Biochem J 266: 625–636

Vermeer C, Jie KSG, Knapen MHJ (1995) Role of vitamin K in bone metabolism. Annu Rev Nutr 15: 1–22
Wang ZM, Gallagher D, Nelson ME, Matthews DE, Heymsfield SB (1996) Total-body skeletal muscle mass: evaluation of 24-h urinary creatinine excretion by computerized axial tomography. Am J Clin Nutr 63: 863–869
Waterlow JC (1984) Protein turnover with special reference to man. Q J Exp Physiol 69: 409–438

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Received July 7, 1997