Plasma Glutathione and Cystathionine Concentrations Are Elevated but Cysteine Flux Is Unchanged by Dietary Vitamin B-6 Restriction in Young Men and Women1,2

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ABSTRACT Cysteine synthesis from homocysteine is catalyzed by two pyridoxal 5′-phosphate (PLP)-dependent enzymes. This suggests that vitamin B-6 status might affect cysteine and glutathione homeostasis, but it is unclear whether this occurs in humans. We assessed the effects of vitamin B-6 status on static and kinetic parameters of cysteine and glutathione metabolism in healthy female (n = 5) and male (n = 4) volunteers (20–30 y) before and after 4 wk of dietary vitamin B-6 restriction (<0.5 mg vitamin B-6/d). Rates of reactions related to cysteine metabolism were measured from blood sampled during primed, constant infusions of [13C5]methionine, [3-13C]serine, and [1H2]cysteine that were conducted after an overnight fast at baseline and after the dietary protocol. Vitamin B-6 restriction reduced the concentration of PLP (55.1 ± 8.3 vs. 22.6 ± 1.3 nmol/L; P = 0.004) and increased concentrations of cystathionine (124%; P < 0.001) and total glutathione (38%; P < 0.008) in plasma. Concentrations of plasma homocysteine, cysteine, cysteinylglycine, and C-reactive protein (an indicator of systemic inflammation) were not affected by dietary vitamin B-6 restriction. The rate of cysteine synthesis via transsulfuration was below detection limits in this protocol. Neither the fractional synthesis rate of cystathionine nor whole-body cysteine flux was affected by vitamin B-6 restriction. These data indicate that glutathione homeostasis is altered by dietary vitamin B-6 deficiency and appears to be unrelated to cysteine flux under conditions of minimal amino acid intake as evaluated in this study. J. Nutr. 136: 373–378, 2006.

KEY WORDS: • cysteine • glutathione • human • transsulfuration • vitamin B-6

Cysteine is a conditionally essential amino acid and a precursor of important compounds, such as glutathione and taurine (1). In addition to the diet, this amino acid is obtained by de novo synthesis from the transsulfuration pathway (Fig. 1). This metabolic pathway might be important for maintenance of cysteine homeostasis under certain conditions, such as during oxidative stress (2) or in response to specific hormones (3,4). Cysteine synthesized by this pathway can be an important contributor to glutathione synthesis (2,5–7). Considering the importance of glutathione as an antioxidant, in detoxification (8), and as a precursor of important compounds, such as glutathione and taurine (1), it is not surprising that many tissues express enzymes that catalyze its synthesis. The rate of cysteine synthesis via transsulfuration in plasma after a methionine load suggests that low vitamin B-6 status reduces flux through the transsulfuration pathway (19–21), but little is known about the effects of vitamin B-6 status on transsulfuration flux in humans under more physiological conditions. In this study, we tested the
hypothesis that low vitamin B-6 status impairs cysteine synthesis by the transsulfuration pathway of humans under steady-state conditions with minimal amino acid intake. In addition to static measures of cysteine, glutathione, and related metabolites in plasma, we utilized [13C5]methionine, [3,13C]serine, and [3H2]cysteine tracers to investigate rates of transsulfuration pathway reactions and cysteine metabolism in humans before and after dietary vitamin B-6 restriction. Also, C-reactive protein concentration was measured in plasma as a preliminary analysis of the effects of controlled vitamin B-6 status on inflammation; its elevation is associated with low vitamin B-6 status in some clinical conditions (22–25).

SUBJECTS AND METHODS

Materials

L-[5,5,5-2H3]leucine, L-[3-13C]serine, L-[3,3-2H3]cysteine, and L-[13C5]methionine were purchased from Cambridge Isotope Laboratories. Tracer solutions were prepared in isotonic saline, filtered sterilized, and analyzed to ensure lack of pyrogenicity and microbial contamination. Pyrogenicity was determined by a commercial laboratory (Focus Technologies) using the Limulus amebocyte lysate assay.

Human subjects

Subjects were healthy, nonsmoking 20- to 30-y-old men and nonpregnant women who did not use medications (including oral contraceptives) that might interfere with vitamin B-6 metabolism and who agreed to abstain from alcohol use during the study period. Subjects were selected for the C/C variant of the methylenetetrahydrofolate reductase 677C>T polymorphism to limit genetic variability (26). All subjects met the inclusion criteria for vitamin status (plasma vitamin B-12 >1.87 ng/L, plasma folate >150 nmol/L, and plasma homocysteine <10 mg/L) at baseline and were nutritionally adequate meals of controlled protein content were consumed by subjects for 2 d before the first infusion. On the day after the first infusion, subjects began consuming the vitamin B-6-deficient diet (<0.5 mg vitamin B-6/d) for 4 wk (27). Subjects consumed breakfast in the GCRC, were given a take-out lunch to eat at their convenience, and returned to the GCRC to consume their evening meal. Vitamin and mineral inadequacies of the study diets (other than vitamin B-6) were compensated for by custom supplements that were administered daily to the subjects. Compliance with the dietary regimen was monitored weekly by measurement of plasma PLP concentration.

Analytical methods

Plasma PLP concentration was measured as the semicarbazone-derivative by reverse-phase HPLC with fluorescence detection (28). The concentrations of folate and vitamin B-12 in plasma were measured with a commercial competitive radiobinding assay (Quantaphase II B-12/Folate radiobinding assay, Bio-Rad). Total concentrations of homocysteine, cysteine, glutathione, and cysteinylglycine in plasma were measured, after reduction by tris(2-carboxyethyl)phosphine hydrochloride and conversion to fluorescent derivatives by reaction with ammonium 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonate, by reverse-phase HPLC with fluorescence detection (29). Cystathionine concentrations in plasma were measured by isotope dilution GC-MS as described previously for methionine (30). Samples and calibration standards contained 250 nmol/L [14CH3]cystathionine as an internal standard, and the calibration curve was derived from the abundances of unlabeled and labeled isotopomers of cystathionine (mass/charge ratio of 678 and 682, respectively). All samples were assayed in a single run (%CV = 4.3). High-sensitivity C-reactive protein assays were determined using an electrochemiluminescent method (Roche Modular System, Roche Diagnostics) by the Clinical Chemistry Laboratory at Shands Hospital, University of Florida. According to this method, normal is <1 mg/L, 1–3 mg/L is borderline elevated, 3–5 mg/L is elevated, and >10 mg/L is indicative of systemic inflammation.

Infusion protocol

The preparation of subjects for the infusion protocol was recently published (30–32). For 7 of 9 subjects, the infusions were initiated at ~0830 with a 5-min, 20-mL priming dose that delivered 9.26, 1.62, 1.00, and 1.87 μmol/kg of [3-13C]serine, [13C5]methionine, [3H2]cysteine, and [3H2]leucine, respectively. The 9-h constant infusion followed immediately after the priming dose, and delivered 20 mL of infusion solution per hour that contained 9.26, 1.62, 1.00, and 1.87 μmol/kg of [3-13C]serine, [13C5]methionine, [3H2]cysteine, and [3H2]leucine, respectively. The 9-h constant infusion followed immediately after the priming dose, and delivered 20 mL of infusion solution per hour that contained 9.26, 1.62, 1.00, and 1.87 μmol/kg of [3-13C]serine, [13C5]methionine, [3H2]cysteine, and [3H2]leucine, respectively. The 9-h constant infusion (Fig. 1). The resulting [13C5]methionine, [3-13C]serine, [3H2]cysteine, and [3H2]leucine, respectively. The 9-h constant infusion solutions were prepared in isotonic saline, filtered sterilized, and analyzed to ensure lack of pyrogenicity and microbial contamination. Pyrogenicity was determined by a commercial laboratory (Focus Technologies) using the Limulus amebocyte lysate assay.

GC-MS analysis of amino acid isotopic enrichments

Plasma free amino acids were isolated and derivatized as previously described (32). Isotopic enrichment was determined by negative chemical ionization GC-MS using a Finnigan-Thermospectro Voyager GC-MS and a 30 m poly(5% diphenyl:95%dimethylsiloxane) fused silica capillary column (Equity 5; Supelco) (32). The abundance of specific ions was measured by selected ion monitoring at the mass/charge ratios described previously (32), with the exception of cysteine, which was measured at the mass/charge ratios of 535–537. Isotopic enrichments are expressed as molar ratios of labeled:nonlabeled isotopomers after correction for the natural abundance of stable isotopomers (33).

Kinetic analyses

The [13C5]methionine and [3,13C]serine tracer paradigms were described in detail previously (32). Briefly, one 13C-atom is lost from [13C5]methionine when it is utilized in the methionine cycle for methyltransferase reactions (Fig. 1). The resulting [13C4]homocysteine can be condensed with serine by cystathionine β-synthase to form...
The plateau enrichments (0.13 mg/L) did not differ from those at baseline (0.71 mg/L/C255Q). The plateau enrichments of the infused [3-13C]serine, [13C]methionine, [4-13C]cysteine, and [1-13C]leucine tracers were calculated as the mean enrichments for the 5–9 h time points. Ep values for the [4-13C]cysteine tracer derived this way did not differ significantly from those derived by fitting enrichment data to single exponential curves defined by the equation $E = E_0 \left(1 - e^{-t}\right)$, where $E_0$ is the enrichment at infinity (i.e., plateau enrichment). Plateau enrichments of labeled [13C]cystathionine and [13C]homocysteine were determined by fitting enrichment data to a single exponential curve. 

Steady-state kinetics of amino acid tracer calculations were calculated using standard equations (33). The flux of cysteine is its rate of appearance from endogenous production (i.e., transsulfuration, glutathione catabolism, and protein breakdown) and the tracer infusion. The flux of cysteine ($Q_{	ext{Cys}}$) in the plasma pool was calculated from the [13C]cystathionine enrichment rate ($I_{	ext{Cys}}$), the [13C]cysteine tracer enrichment ($E_{	ext{Cys}}$), and the [1-13C]cysteine plateau enrichment ($E_{	ext{pCys}}$) as:

$$Q_{	ext{Cys}} = I_{	ext{Cys}} \times \left(E_{	ext{Cys}}/E_{\text{pCys}} + 1\right).$$

Leucine flux ($Q_{\text{Leu}}$) was calculated similarly. Fluxes of serine and methionine were calculated as described previously (32).

Relative flux of cysteine, which is the rate of appearance of cysteine after adjustment for protein breakdown, was calculated by dividing $Q_{	ext{Cys}}$ by $Q_{\text{Leu}}$. The fractional synthesis rate (FSR) of cystathionine was calculated by dividing the initial rate of cystathionine synthesis by the plateau enrichment of its precursor (i.e., [13C]methionine) as follows (32):

$$FSR_{\text{Cys}} = \text{initial rate of Csn tracer infusion}/E_{\text{pCys}}.$$  

The initial rate of cystathionine enrichment was calculated as the slope of the line plotted through the early points (0.5–2 h) of the linear portion of the enrichment curve of [13C]cystathionine.

The results were analyzed using SigmaStat 3.0 (SPSS). Values are means ± SE. Data collected at baseline and after dietary vitamin B-6 restriction were compared by paired t tests. All data were analyzed for differences due to gender by the 2-sample t test. Differences were considered significant at $P < 0.05$.

### RESULTS

As reported in a previous paper on other aspects of this study (30), dietary vitamin B-6 restriction reduced the plasma PLP concentration by 59% (55.1 ± 8.3 vs. 22.6 ± 1.3 nmol/L; $P = 0.004$) (30). All of the postrestriction plasma PLP concentrations (16–29 nmol/L) covered a range from deficient (<20 nmol/L) to marginal vitamin B-6 status (<30 nmol/L). As reported previously (30), folate and vitamin B-12 status were normal before and after dietary vitamin B-6 restriction.

Fasting plasma homocysteine concentration was not affected by dietary vitamin B-6 restriction (6.4 ± 0.4 vs. 6.1 ± 0.4 μmol/L) (30). In contrast, the concentration of cystathionine in plasma was 124% greater ($P < 0.001$) after dietary vitamin B-6 restriction than at baseline (Table 1). Concentrations of cysteine and cysteinylglycine in plasma were not affected by dietary vitamin B-6 restriction, but the concentration of glutathione in plasma was 38% greater ($P = 0.008$) after dietary vitamin B-6 restriction than at baseline.

As previously published, the plateau enrichments of the [13C]methylcysteine (7–8%) and [1-13C]leucine (2.3%) tracers did not differ after dietary vitamin B-6 restriction (30) and were similar to previous reports (31,32). Neither [13C]cystathionine enrichment (4–5%) nor the fractional rate of cystathionine synthesis (FSR) differed after vitamin B-6 restriction compared with baseline. The plateau enrichments of the infused [3-13C]cysteine tracer (~3.7%) did not differ between baseline and dietary vitamin B-6 restriction (Fig. 2). Neither cysteine flux ($Q_{\text{Cys}}$) nor relative cysteine flux ($Q_{\text{Cys}}/Q_{\text{Leu}}$) differed after vitamin B-6 restriction compared with baseline (Table 2).

Plasma C-reactive protein concentrations (range = 0.2–1.3 mg/L) were in the normal range for all subjects at all time points. Values measured after dietary vitamin B-6 restriction (0.56 ± 0.13 mg/L) did not differ from those at baseline (0.71 ± 0.16 mg/L).
Men and women did not differ in any static or kinetic measurement (P > 0.05).

DISCUSSION

It is clear that inhibition of transsulfuration by gene knockout or chemical inhibitors can reduce cysteine and glutathione syntheses in rodents (2,5–7). Similarly, severe vitamin B-6 deficiency reduces the activities of transsulfuration enzymes (10–14,16), interferes with cystathionine catabolism in rats in vivo (34), and yields elevated concentrations of cystathionine in urine (18). These data, combined with those from an investigation of the effects of graded levels of dietary pyridoxine on activities of hepatic transsulfuration enzymes in vitro (16), suggest that cystathionine γ-lyase is the transsulfuration enzyme most sensitive to vitamin B-6 status in rats.

Cystathionine concentrations are elevated in urine of humans after controlled dietary vitamin B-6 restriction (20). In addition, postmethionine load hypercystathioninemia due to low vitamin B-6 status was alleviated in humans by supplemental pyridoxine (19,35). Here we report that a dietary vitamin B-6 restriction that reduces vitamin B-6 status to a marginal (plasma PLP < 30 nmol/L), but not necessarily deficient level (plasma PLP < 20 nmol/L), elevates the concentration of cystathionine in plasma of fasting humans (Table 1). In contrast, this level of dietary vitamin B-6 restriction did not elevate the concentration of homocysteine in plasma. As predicted, the fractional rate of cystathionine synthesis, which reflects in vivo flux through cystathionine β-synthase, was not reduced by dietary vitamin B-6 restriction. Combined, these data strongly suggest that the obstruction of the transsulfuration pathway of humans in moderate vitamin B-6 deficiency is at the point of the cystathionine γ-lyase reaction, and not at the cystathionine β-synthase reaction.

We did not measure the rate of cysteine synthesis from transsulfuration in this study because any production of [13C]cysteine from the [3-13C]serine tracer was below detection limits. Similar results were obtained previously using the current (31,32) and similar (36) combinations of experimental protocol and analytical methodology. Failure to detect [13C]cysteine enrichment might reflect analytical limitations because others have reported transsulfuration rates from related protocols when breath [13C]CO2 measured by more precise isotope ratio MS was used as an indirect measure of transsulfuration flux (33,37). In addition, failure to detect [13C]cysteine enrichment also might be a function of the dietary conditions used. Specifically, subjects fasted for ~12 h beforehand and ingested negligible protein during the 9-h infusion. These conditions favor homocysteine remethylation (methionine conservation) rather than transsulfuration due to low production of S-adenosylmethionine, which is an activator of the transsulfuration pathway as reviewed by Finkelstein (38).

In support of this hypothesis, Storch et al. (33) reported that transsulfuration flux in the postabsorptive state was 52% lower than in the fed state when methionine was consumed within a complete amino acid mixture. Regardless of the cause, the absence of these data precludes formation of definitive statements about the effects of vitamin B-6 status on transsulfuration flux at this time.

Some reports indicate that vitamin B-6 status affects cysteine metabolism. For example, concentrations of cysteine in plasma of rats and pigs fed a vitamin B-6–deficient diet were lower than those in control rats and pigs (11,17,39). However, these effects are not entirely consistent (17), possibly because vitamin B-6 deficiency also affects pyridoxal 5'-phosphate–dependent enzymes involved in the catabolism of cysteine (40,41). Cysteine concentrations in the plasma of adult humans were not affected by 3 wk of vitamin B-6 restriction (42) or by supplementation with 15 mg/d folic acid and 200 mg/d pyridoxine (43), although cysteine concentrations in erythrocytes tripled after supplementation in the latter study. Deficient vitamin B-6 status was associated with elevated plasma cystathionine concentrations but not with abnormal plasma cysteine concentration in asthma patients who were treated with the vitamin B-6 antagonist theophylline. Further, 6 wk of supplemental pyridoxine intake (20 mg/d) significantly reduced plasma cystathionine concentrations in patients and controls in that study, but did not affect plasma cysteine concentration (19).

Neither the concentration of cysteine in plasma nor the flux of cysteine was affected by 4 wk of dietary vitamin B-6 restriction in the present study. There are several possible explanations for this finding. The simplest interpretation is that this level of dietary vitamin B-6 restriction does not significantly affect cysteine homeostasis. This interpretation requires several assumptions that have not been evaluated in vitamin B-6 deficiency: that concentrations of cysteine in plasma are reflective of concentrations within cells; that cysteine flux measured in the absence of dietary protein intake is representative of cysteine flux under other dietary conditions; and that cells do not adapt to a change in cysteine synthesis by increasing entry of cysteine from other labile pools (e.g., from glutathione) or reducing catabolism of cysteine (e.g., to taurine).

Little is known about the effects of vitamin B-6 deficiency on intracellular cysteine concentrations. Some data (43) indicate that intracellular and extracellular concentrations of cysteine in humans do not always change in parallel. Also, cysteine concentrations might be slow to change during vitamin B-6 depletion because of the entry of cysteine from other pools. Data from studies of rat liver and isolated hepatocytes indicate that cysteine is readily removed from the glutathione pool, and diverted from the taurine pool, when cysteine availability is low (44,45). Rapid transfer of cysteine from the glutathione pool to the cysteine pool was confirmed in humans (46). Therefore, cysteine flux might appear unchanged after dietary vitamin B-6 restriction because cysteine entering from the glutathione pool, or kept out of the taurine pool, might counterbalance deficits of cysteine synthesis. Synthesis of taurine from cysteine, which is catalyzed by the PLP-dependent enzyme cysteine sulfinate decarboxylase, is severely depressed in vitamin B-6–deficient rat tissues (41). Therefore, vitamin B-6 deficiency could further reduce catabolism of cysteine by this pathway.

An unexpected finding was the observation that dietary vitamin B-6 restriction caused the concentration of glutathione in plasma to increase by ~40% (Table 1). The cause of this elevation is unclear, but could reflect increased glutathione breakdown in the extracellular compartment. However, concentrations

### TABLE 2

| Fractional synthesis of cystathionine (FSR<sub>con</sub>), cysteine flux (Q<sub>Cys</sub>), and relative cysteine flux (Q<sub>Cys/Q<sub>Lae</sub></sub>) in humans at baseline and after 4 wk of dietary vitamin B-6 restriction (0.05 mg/d)<sup>1</sup> |  |
|---|---|---|
| | FSR<sub>con</sub> %/h | Q<sub>Cys</sub> μmol/(kg·h) | Q<sub>Cys/Q<sub>Lae</sub></sub> |
| Baseline | 15.7 ± 2.4 | 26.1 ± 1.2 | 0.33 ± 0.02 |
| Restricted | 16.1 ± 1.3 | 26.5 ± 0.9 | 0.34 ± 0.02 |

<sup>1</sup> Values are means ± SE; n = 9 for FSR<sub>con</sub>, n = 7 for Q<sub>Cys</sub> and Q<sub>Cys/Q<sub>Lae</sub></sub>.
of cysteinylglycine and cysteine in plasma, which did not differ between baseline and dietary vitamin B-6 restriction, do not support this explanation. Another possible explanation is elevated transport of glutathione from the liver as a source of cysteine for extrahepatic tissues. This would be important if the concentration of cysteine in extrahepatic tissues was reduced by dietary vitamin B-6 restriction.

Another possible explanation for elevated plasma glutathione after dietary vitamin B-6 restriction is that oxidative stress, which is observed in tissues of vitamin B-6–deficient animals (47–49), might have increased glutathione synthesis and efflux from the liver to blood plasma (50–53). Effects of vitamin B-6 deficiency on glutathione synthesis or efflux from liver have not been reported. Another consequence of oxidative stress in liver during vitamin B-6 deficiency would be a redox-mediated elevation of cystathionine β-synthase activity (54), which might have contributed to the elevated plasma cystathionine concentration observed in our subjects.

An early report of the effects of vitamin B-6 deficiency on glutathione metabolism in rats concluded that vitamin B-6 deficiency elevated hepatic glutathione concentrations (10). The most comprehensive investigation of the relation between vitamin B-6 status and glutathione metabolism concluded that there was little or no effect of 8 wk of dietary vitamin B-6 deficiency on glutathione concentrations in liver, kidney, brain, lung, spleen, and plasma of rats, despite significantly reduced activities of hepatic cystathionine β-synthase and cystathionine γ-lyase (13). The extremely high methionine:cysteine ratio (7.5:1) of the experimental diet might have influenced these results by increasing production of S-adenosylmethionine, which is an allosteric activator of cystathionine β-synthase. The difference between the concentrations of glutathione in the plasma of vitamin B-6–deficient rats compared with vitamin B-6–restricted humans suggests a fundamental difference between the 2 model systems.

Data from several observational studies suggest that a negative correlation exists between vitamin B-6 status and the concentration of C-reactive protein in plasma (23,25,55) but the cause of this association is unknown. The small sample size, the marginal level of deficiency reached, and the subject population under investigation all limit interpretation of the current data. Nonetheless, the concentration of C-reactive protein in plasma was not significantly elevated by low vitamin B-6 status in the healthy young adults evaluated in this study. Perhaps a more severe deficiency, or a concurrent chronic inflammatory condition, might be necessary to elevate C-reactive protein levels.

In conclusion, the results of this study provide further evidence that the primary effect of vitamin B-6 deficiency on transsulfuration is reduced cystathionine γ-lyase activity. Further, moderate vitamin B-6 deficiency elevates the concentration of glutathione in the plasma of humans. It is unclear whether the latter observation is related to an effect on transsulfuration because cysteine flux was not significantly different after vitamin B-6 restriction. Further investigation of the mechanism(s) responsible for increased plasma glutathione concentration is warranted. Investigations of the effect of vitamin B-6 status on intracellular metabolism of cysteine and glutathione in animals, as well as tracer investigations conducted in humans during ingestion of physiological amounts of dietary protein, would help clarify the effect of vitamin B-6 status on the homeostasis of cysteine and glutathione.

LITERATURE CITED


