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Vitamin B-6 Restriction Reduces the Production of Hydrogen Sulfide and its Biomarkers by the Transsulfuration Pathway in Cultured Human Hepatoma Cells[1–3]

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Abstract

Background: Pyridoxal 5′-phosphate (PLP) functions as a coenzyme in many cellular processes including one-carbon metabolism and the interconversion and catabolism of amino acids. PLP-dependent enzymes, cystathionine β-synthase and cystathionine γ-lyase, function in transsulfuration but also have been implicated in the production of the endogenous gaseous signaling molecule hydrogen sulfide (H_2S) concurrent with the formation of the biomarkers lanthionine and homolanthionine.

Objective: Our objective was to determine if H_2S production and concurrent biomarker production is affected by vitamin B-6 restriction in a cell culture model.

Methods: We used cultured human hepatoma cells and evaluated static intracellular profiles of amino acids and in vivo kinetics of H_2S biomarker formation. Cells were cultured for 6 wk in media containing concentrations of pyridoxal that represented severe vitamin B-6 deficiency (15 nmol/L pyridoxal), marginal deficiency (56 nmol/L pyridoxal), adequacy (210 nmol/L pyridoxal), and standard medium formulation providing a supraphysiologic pyridoxal concentration (1800 nmol/L pyridoxal).

Results: Intracellular concentrations of lanthionine and homolanthionine in cells cultured at 15 nmol/L pyridoxal were 50% lower ($P < 0.002$) and 47% lower ($P < 0.0255$), respectively, than observed in cells cultured at 1800 nmol/L pyridoxal. Extracellular homocysteine and cysteine were 58% and 46% higher, respectively, in severely deficient cells than in adequate cells ($P < 0.002$). Fractional synthesis rates of lanthionine ($P < 0.01$) and homolanthionine ($P < 0.006$) were lower at 15 and 56 nmol/L pyridoxal than at both higher pyridoxal concentrations. The rate of homocysteine remethylation and the fractional rate of homocysteine production from methionine were not affected by vitamin B-6 restriction. In vitro studies of cell lysates using direct measurement of H_2S also had a reduced extent of H_2S production in the 2 lower vitamin B-6 conditions.

Conclusion: In view of the physiologic roles of H_2S, these results suggest a mechanism that may be involved in the association between human vitamin B-6 inadequacy and its effects on human health. J. Nutr. 144: 1501–1508, 2014.

Introduction

Epidemiologic studies have shown that low dietary intake of vitamin B-6 or low plasma pyridoxal 5′-phosphate (PLP)[7] concentration are independent risk factors for cardiovascular disease, stroke, and venous thrombosis, with little or no relation to plasma homocysteine (1–7). Although the vitamin B-6 coenzyme PLP is necessary in many metabolic processes that may influence disease risk, the mechanism remains unclear.

In view of the many coenzymatic functions of PLP, vitamin B-6 insufficiency has the potential to exert multiple metabolic effects (8–12). Vitamin B-6 insufficiency also can induce both direct and indirect changes in one-carbon metabolism by virtue of its multiple coenzymatic roles. Adequate cellular PLP is needed for optimal functioning of the glycine cleavage system, cytosolic and mitochondrial forms of serine hydroxymethyltransferase, and of transsulfuration enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in one-carbon metabolism. Short-term vitamin B-6 deficiency induced by 28-d dietary restriction in humans increased plasma glycine and

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[3] Supplemental Table 1 is available from the ''Online Supporting Material'' link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
[7] Abbreviations used: ASR, absolute synthesis rate; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; EBSS, Earle's balanced salt solution; FSR, fractional synthesis rate; GCMS, gas chromatography mass spectrometry; HepG2, human hepatoma; H_2S, hydrogen sulfide; PLP, pyridoxal 5′-phosphate; TCA, trichloroacetic acid.
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cystathionine and altered proportions of other one-carbon metabolites (8,13,14). Larger population studies confirmed the inverse relation of plasma cystathionine and vitamin B-6 status (15). Severe deficiency in rats also affected concentrations of homocysteine, S-adenosylmethionine, and glutathione (11,12,16–18).

The transsulfuration pathway mediates cysteine production from homocysteine catabolism and contributes to the endogenous production of hydrogen sulfide (H_2S) (19–21). The transsulfuration enzymes CBS and CSE both produce H_2S concurrent with the formation of the biomarkers lanthionine and homolanthionine (19,20). Jurkowska et al. (22) showed that both enzymes contribute substantially to cysteine desulfhydration in primary hepatocytes and that increased H_2S production corresponds with higher lanthionine concentrations in media. H_2S is a gaseous signaling molecule (23–26) that modulates physiologic functions including relaxation of smooth muscle by opening K_{ATP} channels (27–31). H_2S can attenuate myocardial ischemia-reperfusion injury by protecting mitochondrial function (32). Mice with CSE deleted have reduced concentrations of H_2S in serum, heart, aorta, and other tissues, along with hypertension and reduced vasorelaxation (33). Because of its ability to modulate vasodilation, H_2S was identified as a cardioprotective agent. Direct quantification of H_2S is difficult and sometimes inaccurate because of its rapid oxidation and volatility (34). Therefore, the simultaneous production of the biomarkers lanthionine and homolanthionine with H_2S could provide an indirect marker reflective of H_2S production (20).

This study employed a human hepatoma (HepG2) cell model to determine the influence of vitamin B-6 in the culture medium on the kinetics of H_2S biomarker production, the patterns of H_2S biomarkers and other metabolites associated with the transsulfuration pathway, and the capacity to produce H_2S. This cell culture model was used to facilitate experimentation in which cellular PLP concentrations can be manipulated and strictly maintained by culturing in various concentrations of vitamin B-6.

Materials and Methods

Materials. The HepG2 cell line was obtained from the American Type Culture Collection. Modified MEM with Earle's balanced salt solution (EBSS) was obtained as the standard formulation containing ~1800 nmol/L pyridoxal and in custom form (without pyridoxal) from HyClone. All other culture medium supplements were purchased from Hyclone or Cellgro. Amino acids used for calibration were purchased from Sigma Aldrich, whereas [U-$^{13}C_5$] L-methionine (97–98% purity) and [3,3-D_2] L-cysteine (98% purity) were obtained from Cambridge Isotope Laboratories. All other HPLC-grade chemicals and solvents were purchased from Fisher Scientific or Sigma Aldrich.

Cell culture. After initial culturing for 2 wk in complete MEM/EBSS, cultures were transferred to 75-cm^2 flasks and maintained on the custom vitamin B-6–deficient MEM/EBSS supplemented with 4 different concentrations of pyridoxal: 15 nmol/L pyridoxal represented severe deficiency, 56 nmol/L pyridoxal represented marginal deficiency, 210 nmol/L pyridoxal represented adequate vitamin B-6 status, and 1800 nmol/L pyridoxal, which was equivalent to the pyridoxal concentrations found in commercial media. We used pyridoxal as the major source of vitamin B-6 provided to cells because pyridoxal is the primary form taken up by cells from the plasma despite the fact that PLP bound to albumin is the major circulating form of the vitamin (35). FBS used in this study contributed 14.8 nmol/L pyridoxal and 0.807 nmol/L PLP. Stated pyridoxal concentrations in experimental media reflected the contribution of FBS and added pyridoxal. All complete media contained 2 mmol/L L-glutamine, 0.1 mmol/L each nonessential amino acid, and 1 mmol/L sodium pyruvate. These media also contained added penicillin (87,000 IU/L), streptomycin (87 mg/L), and amphotericin B (217 μg/L) provided by a stock antibiotic/antimycotic solution (Cellgro) and 10% FBS. Cells were incubated in 5% CO_2 at 37°C and passaged every 3–4 d depending on confluency for 6 wk, which yielded a steady state of intracellular PLP concentration as described below.

PLP and amino-thiol analysis. Intracellular PLP in cultured cells was determined weekly. Harvested cells were washed with PBS and sonicated for 45 s in a 1.5-mL cold deionized water suspension. A portion of the resulting cell lysate (500 μL) was immediately combined with 500 μL of 10% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,600 × *g* for 10 min. The PLP concentration was then determined by HPLC (Dionex) with fluorescence detection after semicarbazide derivatization (36). The values for PLP and other metabolites were expressed per milligram cellular protein, with protein determined by analysis of another aliquot of the lysate (100 μL) (without TCA) (37). All data are from 4 independent cultures and are reported as means ± SDs (n = 4).

Amino-thiol concentrations (i.e., total homocysteine, cysteine, and glutathione) were measured as ammonium 7-fluorescence-2-oxa-1,3-diazole-4-sulfonate derivatives by reversed-phase HPLC with fluorescence detection (38). Cell lysates and media were treated with a reducing agent, tris(2-carboxyethyl)phosphine, to reduce disulfide species and allow quantification of total concentration for each amino-thiol. Intracellular homocysteine is strictly regulated by export mechanisms, and under normal conditions considerable amounts are exported (39,40); therefore, the extracellular total homocysteine concentration reflects cellular function.

Determination of amino acid concentration. We quantified methionine, cystathionine, lanthionine, and homolanthionine by gas chromatography mass spectrometry (GCMS) (Thermo DSQII) as N-propyl ester, heptafluorobutyramide derivatives in negative chemical ionization mode with selected-ion monitoring (41,42). Lanthionine and homolanthionine peaks were measured relative to a norleucine internal standard, whereas methionine and cystathionine were quantified relative to [$^{13}C_5$]-methionine and [D_4]-cystathionine internal standards (13,14). Extractions were performed by sonication of cells in 50 μL of 66% (w/v) TCA. Because homolanthionine is not commercially available, the homolanthionine reference standard was prepared enzymatically by using recombinant human CSE (20) and quantified relative to the cystathionine response curve.

In vivo kinetics of H_2S biomarker synthesis. HepG2 cells that had been cultured as described above to ~90% confluency were incubated for 24 h in the respective fresh media. The media were replaced with fresh portions enriched with [U-$^{13}C_5$] L-methionine and [3,3-D_2] L-cysteine, which was considered time 0. Cells and samples of media were collected at 0-, 0.5-, 1-, 2-, 4-, and 6-h time points. Cells were lysed immediately by the addition of TCA to a final concentration of 10% (w/v) solution, followed by sonication and addition of methanol for deproteination, and then stored at −80°C until analysis.

Isotopic enrichment was determined as described above by using GCMS with selected-ion monitoring for labeled and unlabeled species of methionine, homocysteine, cysteine, lanthionine, and homolanthionine. For cysteine and homocysteine, this analysis allowed determination of isotopic enrichment of the reduced form (i.e., nondisulfide) only, which we assumed was representative of enrichment of the total pools. All enrichment values were corrected for natural abundance expressed as the molar ratio of labeled amino acid divided by the sum of unlabeled + labeled forms. Plateau enrichments of tracers were estimated by fitting enrichment data to single exponential curves defined by $E = E_f(1-e^{-kt})$, where E is the enrichment at time t (h), E_f is the enrichment plateau, and k is the rate constant (h^{-1}). The fractional synthesis rate (FSR) for lanthionine and homolanthionine, which reflects the fraction of the total pool that is synthesized per hour, was calculated as $FSR = IR/E_{precursor}$, where IR is the initial rate (i.e., slope) of enrichment estimated from 0 to 1 h. $E_{precursor}$ was the measured plateau enrichment of the labeled precursor amino acid (i.e., [$^{13}C_4$]-homocysteine for homolanthionine and [D_2]-cysteine for lanthionine). Apparent absolute synthesis rates

(ASRs) also were estimated by using ASR = FSR × [AA], where [AA] is the total concentration (intracellular + extracellular) of the amino acid (i.e., homolanthionine or lanthionine).

H_2S production capacity of HepG2 cell lysates. H_2S producing capacity of cells maintained at different concentrations of pyridoxal was measured as described previously for tissue homogenates (21). Briefly, cells from each vitamin B-6 condition were lysed in 100 mmol/L HEPES, pH 7.4, to obtain a lysate concentration of 100 mg of cell mass/mL. H_2S production reactions were prepared in polypropylene syringes and contained 200 μL cell lysate, 10 mmol/L L-cysteine, and 10 mmol/L L-homocysteine in a total reaction volume of 400 μL. Syringes were made anaerobic by flushing the headspace with nitrogen by using a 3-way stopcock, and maintained in nitrogen in a final total volume (aqueous + gas) of 20 mL by using sleeve stoppers attached to the stopcocks. Reactions were incubated at 37°C with gentle shaking (75 rpm) for 10 min. Control reactions lacking cell lysate were prepared in parallel. The gas phase of the reaction syringes was analyzed by GC (Hewlett Packard) equipped with a 355 sulfur chemiluminescence detector (Agilent). Aliquots of 0.2 mL of the gas phase were aspirated by using gas-tight syringes and injected onto a DB-1 column (30 m × 0.53 mm × 1.0 μm). The flow rate of the carrier gas, helium, was 1 mL/min, and a temperature gradient was from 30°C to 110°C. H_2S content in the injected volume was quantified from the peak areas by using a standard curve generated from H_2S standard gas (Cryogenic Gases).

Statistical analysis. All data were summarized and presented as means ± SDs (n = 4). Overall significant differences among metabolite concentrations were determined by 1-factor ANOVA, followed with Tukey's method for pairwise comparisons and adjusted P values. All data were logarithm (base 10) transformed to meet the Gaussian assumption. Statistical significance was determined at $P < 0.05$. All analyses were performed by using SAS 9.3 and SigmaPlot 12.0. The estimation of plateau enrichments was performed by fitting the data to single exponential regression by using the function of "exponential rise to maximum" or linear regression in SigmaPlot 12.0.

Results

Influence of intracellular PLP concentrations on sulfur amino acid metabolite concentrations. Weekly measurements of intracellular PLP concentrations indicated a steady state by the end of 3 wk of culturing for all experimental groups. Cell cultures were monitored for an additional 3 wk until experimental analysis. PLP concentrations were significantly different between groups ($P < 0.006$) and increased in nonlinear fashion with increasing concentration of pyridoxal in culture medium (**Table 1**). Thus, this protocol was effective in achieving varied intracellular concentrations of PLP and allowed investigation of the metabolic consequences of vitamin B-6 restriction. Cells cultured in the different pyridoxal concentrations exhibited no apparent differences in morphology or growth rate.

Extracellular total homocysteine concentrations (when normalized per milligram of cell protein) were on the order of 40–60 times higher than intracellular total homocysteine concentrations, reflecting extensive homocysteine export. Extracellular total homocysteine concentration was ~35% higher at 15 nmol/L pyridoxal than at 56 nmol/L pyridoxal, and cells in both of the lower pyridoxal treatments exhibited extracellular total homocysteine that was almost twice that of the 210 nmol/L pyridoxal and 1800 nmol/L pyridoxal treatments (adjusted $P < 0.0001$) (**Table 2**). The effect of vitamin B-6 status on intracellular total homocysteine was qualitatively similar to that seen for extracellular total homocysteine, but the concentrations were much lower and differences between treatments were not significant ($P = 0.26$).

The extracellular concentration of total cysteine was ~20% higher in the 15 nmol/L pyridoxal cells than in 56 nmol/L pyridoxal cells (adjusted $P = 0.0002$) and almost 50% higher than 210 and 1800 nmol/L pyridoxal cells (adjusted $P < 0.0001$) (Table 2). These data suggest that cysteine formation by the transsulfuration pathway was maintained even in low vitamin B-6 treatment; however, the elevated cysteine concentrations in 15 nmol/L pyridoxal could also indicate a reduction in utilization. For total glutathione, all intracellular concentrations exceeded those of extracellular total glutathione by a factor of at least 100. There was no difference among pyridoxal treatments in extracellular total glutathione concentration; however, for intracellular total glutathione, the 15 nmol/L pyridoxal group had a 25–30% lower concentration than those of the 56 and 210 nmol/L pyridoxal groups (adjusted $P = 0.0008$) even though the concentration of intracellular cysteine was not depressed by pyridoxal restriction. Thus, we conclude that the lower intracellular total glutathione was not due to a limitation of the precursor cysteine. Similar trends for all analytes were found for the sums of intracellular + extracellular (i.e., total) fractions (**Supplemental Table 1**).

The concentration of intracellular cystathionine, which is governed by the activities of both CBS and CSE, did not exhibit significant differences among pyridoxal treatments. However, the 15 nmol/L pyridoxal treatment exhibited a nonsignificantly ~30% lower cystathionine concentration than the 56 nmol/L pyridoxal treatment (adjusted $P < 0.06$) (Table 2), and the 210 and 1800 nmol/L pyridoxal treatments had ~17% and 26% lower cystathionine concentrations than the 56 nmol/L pyridoxal treatment, respectively. There was no significant difference in cystathionine concentration between the 210 and 1800 nmol/L pyridoxal treatments. Extracellular cystathionine concentrations were 5 times lower than intracellular concentrations. Only the 15 nmol/L pyridoxal treatment was significantly lower than the 1800 nmol/L pyridoxal treatment (adjusted $P < 0.015$).

The intracellular homolanthionine concentration greatly exceeded that of lanthionine by a factor of ~135 for the 15 nmol/L pyridoxal cells vs. a factor of ~80 for all of the higher levels of vitamin B-6 status. Both homolanthionine and lanthionine concentrations were lower in cells with restricted vitamin B-6 status (i.e., 15 and 56 nmol/L pyridoxal treatments) than for the 2 higher pyridoxal concentrations. Intracellular lanthionine concentration was 10 times lower in 15 nmol/L pyridoxal and 56 nmol/L pyridoxal treatments than the 2 higher pyridoxal treatments (adjusted $P < 0.002$) (Table 2), whereas intracellular homolanthionine concentration was 50% lower in the 15 nmol/L pyridoxal treatment than in the 1800 nmol/L pyridoxal treatment (adjusted $P < 0.02$) (Table 2). Extracellular lanthionine concentration was 60% lower in the 15 nmol/L pyridoxal and 56 nmol/L pyridoxal treatments than the 1800 nmol/L pyridoxal treatment (adjusted $P < 0.012$). Extracellular homolanthionine concentrations were 6 times higher than

TABLE 1 Intracellular PLP concentrations of cells cultured for 6 wk in media of different pyridoxal concentrations[1]

Pyridoxal in medium (nmol/L)	Intracellular PLP
	pmol/mg cell protein
15	52.5 ± 2.16[d]
56	104 ± 3.74[c]
210	123 ± 5.53[b]
1800	143 ± 6.45[a]

[1] Values are means ± SDs, n = 4. Means without a common letter differ, $P < 0.05$. PLP, pyridoxal 5′-phosphate.

TABLE 2 Intracellular and extracellular transsulfuration metabolite concentrations in HepG2 cells cultured with different pyridoxal concentrations[1]

	Pyridoxal in medium (nmol/L)			
Metabolite	15	56	210	1800
Extracellular				
Homocysteine, *nmol/mg cell protein*	13.2 ± 1.56[a]	9.78 ± 0.432[b]	5.13 ± 0.572[c]	5.93 ± 0.505[c]
Glutathione, *nmol/mg cell protein*	1.02 ± 0.222	1.06 ± 0.249	1.07 ± 0.140	1.17 ± 0.099
Cysteine, *nmol/mg cell protein*	59.1 ± 6.23[a]	35.7 ± 1.99[b]	29.9 ± 3.63[b]	33.6 ± 2.56[b]
Cystathionine, *nmol/mg cell protein*	0.93 ± 0.189[b]	1.21 ± 0.224[a,b]	1.19 ± 0.159[a,b]	1.58 ± 0.363[a]
Lanthionine, *pmol/mg cell protein*	0.0289 ± 0.00575[a]	0.0256 ± 0.00483[a]	0.0462 ± 0.0123[a,b]	0.0808 ± 0.0351[b]
Homolanthionine, *nmol/mg cell protein*	0.596 ± 0.160	0.664 ± 0.0942	0.767 ± 0.190	0.960 ± 0.191
Methionine,[2] *nmol/mg cell protein*	5.140 ± 0.820[c]	6.87 ± 0.524[b]	7.08 ± 0.511[b]	9.85 ± 1.99[a]
Intracellular				
Homocysteine, *nmol/mg cell protein*	0.217 ± 0.128	0.209 ± 0.075	0.121 ± 0.036	0.142 ± 0.052
Glutathione, *nmol/mg cell protein*	129 ± 14.1[b]	174 ± 15.8[a]	168 ± 12.1[a]	153 ± 4.60[a]
Cysteine, *nmol/mg cell protein*	61.9 ± 3.44[a]	50.3 ± 3.63[b]	55.3 ± 2.92[a]	52.3 ± 2.35[a]
Methionine, *nmol/mg cell protein*	1.21 ± 0.254[b]	1.45 ± 0.176[a,b]	2.25 ± 0.152[a,b]	4.13 ± 1.65[a]
Cystathionine, *nmol/mg cell protein*	4.24 ± 0.215	5.50 ± 0.385	4.71 ± 0.567	4.38 ± 0.793
Lanthionine, *pmol/mg cell protein*	0.698 ± 0.106[c]	1.38 ± 0.179[b]	1.56 ± 0.181[a]	1.70 ± 0.594[a]
Homolanthionine, *pmol/mg cell protein*	93.6 ± 15.8[b]	119 ± 16.8[a,b]	125 ± 26.2[a,b]	177 ± 66.0[a]

[1] Values are means ± SDs, $n = 4$. Means without a common letter differ, $P < 0.05$. HepG2, human hepatoma.
[2] Media methionine concentration = 89.6 ± 8.44 μmol/L.

intracellular concentrations, although there were no differences among cells from the various culturing media. We observed that small quantities of homolanthionine were produced spontaneously during the derivatization of homocysteine standards, and the amount produced increased in a linear relation with the concentration of homocysteine. However, cellular concentrations of homolanthionine were 50 times higher than the amount of homolanthionine produced nonenzymatically during the analytical derivatization procedure.

Vitamin B-6 status affects in vivo kinetics of homolanthionine and lanthionine synthesis in cultured HepG2 cells. In studies of cells incubated with [$^{13}C_5$] L-methionine and [D_2] L-cysteine, time course plots of intracellular [$^{13}C_5$] L-methionine and its remethylation product, [$^{13}C_4$]-methionine, had an enrichment plateau at ~30 min, whereas [D_2] L-cysteine increased in isotopic enrichment throughout the 6-h time course (**Fig. 1**). The plateau enrichment of [$^{13}C_5$] L-methionine did not differ among the 4 pyridoxal groups. The estimated plateau enrichment of [D_2]-cysteine in 56 nmol/L pyridoxal cells was 40% higher than in other pyridoxal groups, possibly because of the lower intracellular and extracellular cysteine concentrations seen at 56 nmol/L pyridoxal.

The appearance of the labeled products [$^{13}C_4$]-homocysteine, [$^{13}C_4$]-cystathionine, and [$^{13}C_4$]-homolanthionine derived from [$^{13}C_5$] L-methionine was readily detected. The plateau enrichment of [$^{13}C_4$]-homocysteine derived from [U-$^{13}C_5$] L-methionine plus [$^{13}C_4$]-methionine (derived from the remethylation of [$^{13}C_4$]-homocysteine) was reached at 2 h (**Fig. 2**). The FSR of homocysteine did not differ significantly among the groups of pyridoxal (**Table 3**). The FSR of cystathionine was significantly higher in the 56 nmol/L pyridoxal treatment than in all other treatments (adjusted $P < 0.05$) (Table 2). Homolanthionine FSR values for all vitamin B-6 concentrations were much lower than those observed for cystathionine, homocysteine, and lanthionine. For example, the FSR values for homolanthionine were ~20–40 times lower than the corresponding FSR values for lanthionine, although the steady-state concentration of homolanthionine greatly exceeded that of lanthionine (Table 2), as stated above. However, vitamin B-6 status did affect the FSR of homolanthionine, with the 210 and 1800 nmol/L pyridoxal

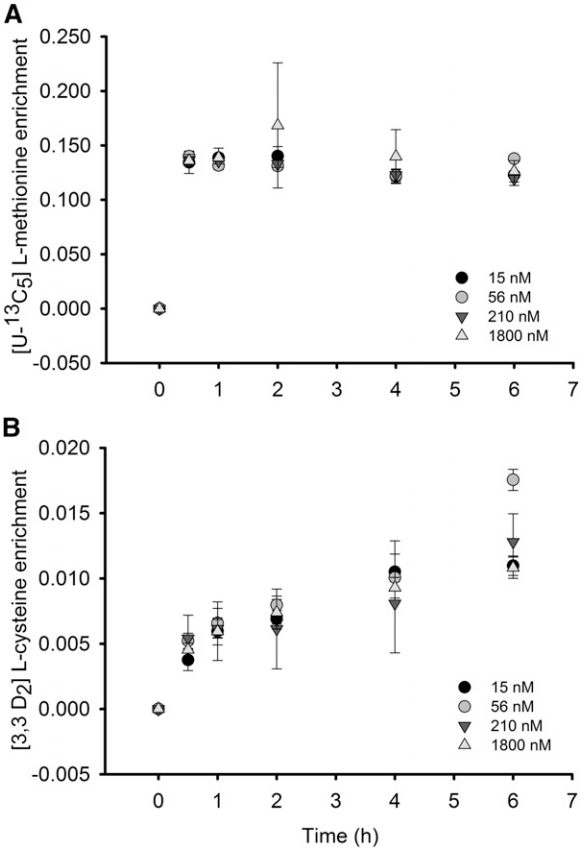

FIGURE 1 The 6-h enrichment curves of [U-$^{13}C_5$] L-methionine (*A*) and [3,3 D_2] L-cysteine (*B*) in HepG2 cells cultured with different concentrations of pyridoxal. Data points are means ± SDs, $n = 4$ experiments. HepG2, human hepatoma.

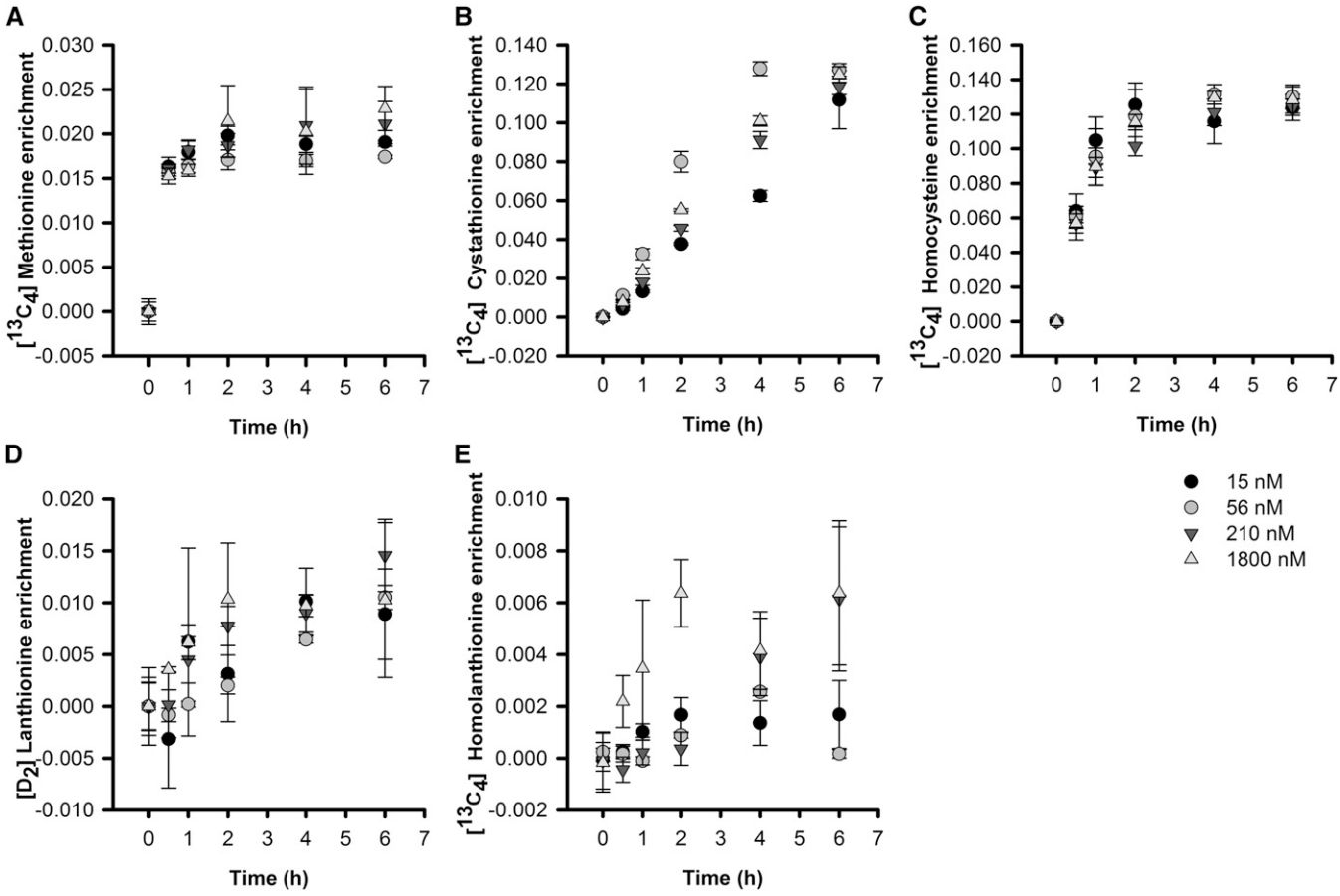

FIGURE 2 The 6-h enrichment curves of amino acid products in HepG2 cells cultured with different pyridoxal concentrations: [$^{13}C_4$] methionine (*A*), [$^{13}C_4$] cystathionine (*B*), [$^{13}C_4$] homocysteine (*C*), [D_2] lanthionine (*D*), and [$^{13}C_4$] homolanthionine (*E*). Data points are means ± SDs, $n = 4$ experiments. HepG2, human hepatoma.

treatments showing a significantly higher homolanthionine FSR than the 15 and 56 nmol/L pyridoxal treatments (adjusted $P < 0.03$) (Table 2).

These kinetic studies focused on the rate of synthesis of [$^{13}C_4$]-homolanthionine and [D_2]-lanthionine from their respective precursors ([$^{13}C_4$]-homocysteine and [D_2] L-cysteine). We detected trace amounts of the forms of homolanthionine and lanthionine derived from the coupling of 2 molecules of the respective labeled precursors to form [$^{13}C_8$]-homolanthionine and [D_4]-lanthionine isotopomers. These were quantitatively minor and below our limits of GCMS quantification and, thus, were not considered in the kinetic analysis.

The estimation of apparent ASR, calculated by using total (sum of intracellular + extracellular) concentrations (Supplemental Table 1), allowed assessment of rates that the total quantity of H_2S biomarkers were produced in comparison to cystathionine (**Table 4**). These data showed that the total production of cystathionine exceeded that of homolanthionine and lanthionine by 2–3 orders of magnitude. In addition, these in vivo data indicated that cells at the 2 lower vitamin B-6 treatments exhibited markedly lower production of these H_2S-specific biomarkers. Because the production of homolanthionine and lanthionine coincide with equimolar amounts of H_2S (19), these data suggest that in vivo H_2S synthesis depends strongly on vitamin B-6 status.

H_2S production capacities in cells related to pyridoxal concentrations in culture media. We also measured the production of H_2S by lysates of cultured cells. When incubated with high concentrations of L-cysteine and L-homocysteine (10 mmol/L), the quantity of H_2S produced was significantly higher in 210 and 1800 nmol/L pyridoxal treatments than in 15 and 56 nmol/L

TABLE 3 Fractional synthesis rates in HepG2 cells cultured with different pyridoxal concentrations[1]

Precursor	Product	15 nmol/L	56 nmol/L	210 nmol/L	1800 nmol/L
		h^{-1}	h^{-1}	h^{-1}	h^{-1}
[$^{13}C_5$]-Methionine	[$^{13}C_4$]-Homocysteine	0.941 ± 0.137	0.816 ± 0.173	0.733 ± 0.0195	0.708 ± 0.0816
[$^{13}C_4$]-Homocysteine	[$^{13}C_4$]-Cystathionine	0.135 ± 0.0134^{c}	0.243 ± 0.00488^{a}	0.145 ± 0.00552^{c}	0.173 ± 0.00719^{b}
[$^{13}C_4$]-Homocysteine	[$^{13}C_4$]-Homolanthionine	0.00662 ± 0.00221^{b}	0.00487 ± 0.000971^{b}	0.00999 ± 0.00364^{a}	0.0287 ± 0.0224^{a}
[D_2]-Cysteine	[D_2]-Lanthionine	0.245 ± 0.0788^{b}	0.106 ± 0.0140^{b}	0.298 ± 0.119^{a,b}	0.697 ± 0.307^{a}

[1] Values are means ± SDs, $n = 4$. Means without a common letter differ, $P < 0.05$. HepG2, human hepatoma.

TABLE 4 Absolute synthesis rates in HepG2 cells cultured with different pyridoxal concentrations[1]

Precursor	Product	15 nmol/L	56 nmol/L	210 nmol/L	1800 nmol/L
		pmol · mg protein^{-1} · h^{-1}	*pmol · mg protein^{-1} · h^{-1}*	*pmol · mg protein^{-1} · h^{-1}*	*pmol · mg protein^{-1} · h^{-1}*
[$^{13}C_4$]-Homocysteine	[$^{13}C_4$]-Cystathionine	700 ± 69.2^{d}	1630 ± 32.7^{a}	854 ± 32.5^{c}	1030 ± 42.9^{b}
[$^{13}C_4$]-Homocysteine	[$^{13}C_4$]-Homolanthionine	4.57 ± 1.52^{b}	3.82 ± 0.761^{b}	8.92 ± 3.25^{b}	32.6 ± 25.6^{a}
[D_2]-Cysteine	[D_2]-Lanthionine	0.178 ± 0.0572^{c}	0.149 ± 0.0196^{c}	0.480 ± 0.192^{b}	1.25 ± 0.548^{a}

[1] Values are means ± SDs, n = 4. Means without a common letter differ, $P < 0.05$. HepG2, human hepatoma.

pyridoxal treatments (adjusted $P < 0.001$) (**Fig. 3**). This finding concurred with the observed differences in apparent ASRs of homolanthionine and lanthionine and indicated that H_2S production capacity is impaired in vitamin B-6 insufficiency.

Discussion

This study quantified steady-state concentrations and in vivo kinetics of transsulfuration pathway constituents with the main focus on the H_2S biomarkers, homolanthionine and lanthionine, in cultured cells of varying intracellular PLP concentration. We, and other studies, have previously reported that controlled vitamin B-6 restriction in humans causes elevation of cystathionine in plasma (13,14) and urine (11), and vitamin B-6 restriction in rats also causes elevation of cystathionine in serum (18) and liver (16). This cell culture model was used because it allows precise control of cellular vitamin B-6 status and determination of the in vivo kinetics and profiles of metabolic products. This protocol yielded intracellular PLP concentrations for the deficient (15 nmol/L pyridoxal) cells that were ~57% and ~63% lower than vitamin B-6–adequate (210 nmol/L pyridoxal) and 1800 nmol/L pyridoxal supraphysiologic conditions, respectively. The relevance of this model is illustrated by the similarity of these results to the relative differences of rat liver PLP concentrations in dietary restriction studies reported previously (11,16).

The biosynthesis of lanthionine is catalyzed primarily by CBS, whereas homolanthionine production is catalyzed solely by CSE (20). Our results show that homolanthionine concentration greatly exceeded that of lanthionine, which indicates that the CSE-catalyzed γ-replacement reaction involving 2 molecules of homocysteine greatly exceeded the formation of lanthionine by

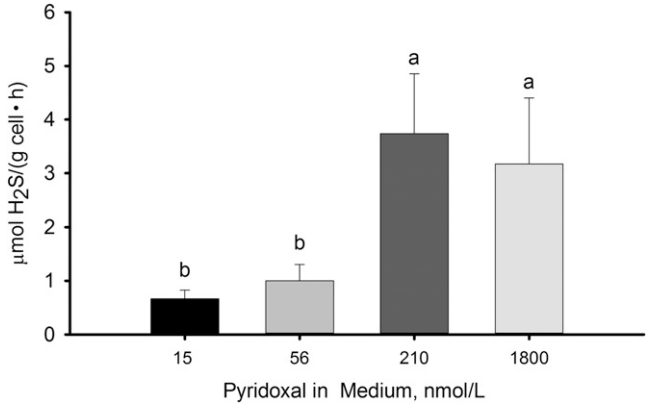

FIGURE 3 H_2S production capacity from HepG2 cells cultured with different pyridoxal concentrations. Data are means ± SDs, n = 4 experiments. The mean of each column without a common letter differs significantly (adjusted $P < 0.001$). HepG2, human hepatoma; H_2S, hydrogen sulfide.

both CSE and CBS (21). Turnover numbers for physiologic substrate (cysteine) concentrations showed CBS produced 4 orders of magnitude more lanthionine than CSE and that the homolanthionine turnover rate increased when homocysteine was elevated (19). The FSRs observed for homolanthionine ranged from 0.00487 to 0.0287 h^{-1} (i.e., ~0.5–2.9% of the pool/h) and were actually lower than those seen for lanthionine (0.106–0.697 h^{-1} or 10.6–69.7% of the pool/h). Additionally informative were the estimates of apparent ASR, in which the ASR for homolanthionine exceeded that of lanthionine by a factor of ~20 for all vitamin B-6 concentrations. For both homolanthionine and lanthionine, the 2 lower vitamin B-6 conditions exhibited lower ASR values and, thus, would be expected to have comparably lower rates of H_2S production because of vitamin B-6 insufficiency. Because 1-mol equivalent of H_2S is produced concurrent with the synthesis of homolanthionine and lanthionine, then quantification of these biomarkers provides an indirect indication of H_2S production. It should be noted that substantial amounts of H_2S are also produced by the CBS-catalyzed condensation of cysteine and homocysteine, yielding cystathionine (19), as well as through the production of pyruvate from cysteine catalyzed by CSE (20).

We have examined the activity of CBS and CSE in the liver of rats fed graded concentrations of dietary vitamin B-6 (16). In that study, we found little or no effect of dietary vitamin B-6 deficiency on CBS activity in the presence or absence of stimulation by S-adenosylmethionine. However, CSE exhibited 70% lower activity in deficient vs. B-6–adequate rats, which was similar to the magnitude of change in liver PLP (16). In vitro activity assays of CBS and CSE were not conducted in the present study. However, the observed reduction in intracellular PLP (Table 1) and the observed ASR values (Table 4) suggest that CSE may have been impaired in the vitamin B-6 insufficiency conditions of the 15 and 56 nmol/L pyridoxal treatments. Because CSE plays a major role in the production of H_2S in some tissues (19,22), our current results suggest that, because of the sensitivity of CSE to inactivation by loss of the PLP coenzyme in vitamin B-6 deficiency, the production of H_2S by this enzyme may be impaired. The direct measurement of H_2S synthesis capacity by cell extracts incubated with high concentrations of cysteine and homocysteine (Fig. 3) provides further evidence of impaired cellular ability to produce H_2S because of vitamin B-6 restriction.

Low intake of vitamin B-6 and low plasma PLP concentration have been shown to be independent risk factors for cardiovascular disease (1–7). Although many hypotheses have been proposed, no mechanistic connection was found. Endogenously produced H_2S was identified as a vasodilator and smooth muscle relaxant (29,33,43). Human studies have shown that patients with coronary heart disease had significantly lower H_2S concentrations than normal controls, which suggests perturbed synthesis and may correlate with disease severity (44). The findings reported here indicate a relation between vitamin B-6 status

and H_2S production, which provides preliminary evidence for a plausible molecular mechanism linking vitamin B-6 insufficiency to vascular disease.

Acknowledgments

J.F.G., B.N.D., and R.B. designed the research; B.N.D., M.A.R., and O.K. conducted the research; B.N.D., O.K., R.B., J.F.G., and Y.-Y.C. analyzed the data; and B.N.D. and J.F.G. wrote the paper. All authors read and approved the final manuscript.

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