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¹³C-Enrichment at carbons 8 and 2 of uric acid after ¹³C-labeled folate dose in man

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Abstract

To evaluate folate-dependent carbon incorporation into the purine ring, we measured ¹³C-enrichment independently at C₂ and C₈ of urinary uric acid (the final catabolite of purines) in a healthy male after an independent oral dose of [6*RS*]-5-[¹³C]-formyltetrahydrofolate ([6*RS*]-5-H¹³CO-H₄folate) or 10-H¹³CO-7,8-dihydrofolate (10-H¹³CO-H₂folate). The C₂ position was ¹³C-enriched more than C₈ after [6*RS*]-5-H¹³CO-H₄folate, and C₂ was exclusively enriched after 10-H¹³CO-H₂folate. The enrichment of C₂ was greater from [6*RS*]-5-H¹³CO-H₄folate than 10-H¹³CO-H₂folate using equimolar bioactive doses. Our data suggest that formyl C of [6*RS*]-10-H¹³CO-H₄folate was not equally utilized by glycinamide ribotide transformylase (enriches C₈) and aminoimidazolecarboxamide ribotide (AICAR) transformylase (enriches C₂), and the formyl C of 10-H¹³CO-H₂folate was exclusively used by AICAR transformylase. 10-HCO-H₂folate may function *in vivo* as the predominant substrate for AICAR transformylase in humans.

Keywords

Folate; Stable isotope; Purine biosynthesis; Uric acid; Human; Mass spectrometry; AICAR transformylase; GAR transformylase; Dihydrofolate reductase; 10-Formyldihydrofolate

Introduction

Two folate-dependent enzymes catalyze the incorporation of carbons (C) into the purine ring in purine nucleotide biosynthesis *de novo* (PNB *de novo*) for the subsequent production of adenine and guanine nucleotides [1]. Glycinamide ribotide (GAR) and aminoimidazolecarboxamide ribotide (AICAR) transformylases insert a C at the positions 8

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(C₈) and 2 (C₂) of the ring, respectively, by using the formyl C of the biologically active [6R]-10-formyltetrahydrofolate ([6R]-10-HCO-H₄folate) [1]. Mainly in microorganisms or birds, the ratio of labeled formate incorporation into the C₂ to C₈ positions has been reported to be about 1.0 [2].

We recently found that ¹³C-formate was incorporated more into the C₂ than C₈ position in humans, even though ¹³C-formate is metabolized to [6R]-10-H¹³CO-H₄folate, which is the common substrate for both transformylases [2]. Therefore, in the present study, we tested whether there are differences in the incorporation of the formyl ¹³C of [6RS]-5-H¹³CO-H₄folate into the C₂ to C₈ positions in an adult male. Metabolism of 5-HCO-H₄folate to 10-HCO-H₄folate is catalyzed by 5,10-methenyltetrahydrofolate (5,10-CH=H₄folate) synthetase producing 5,10-CH=H₄folate, which is then hydrolyzed enzymatically by 5,10-CH=H₄folate cyclohydrolase or non-enzymatically to 10-HCO-H₄folate [3]. To our knowledge, no *in vivo* human data exist for the independent incorporation of the formyl C of 10-HCO-H₄folate into these positions. We developed a liquid-chromatography mass spectrometry (LC/MS/MS) method to independently measure ¹³C-enrichment at C₂ and C₈ of urinary uric acid, the final catabolite of purines in humans [2,4]. Furthermore, 10-[¹³C]-formyl-7,8-dihydrofolate (10-H¹³CO-H₂folate), a substrate utilized by AICAR transformylase but not by GAR transformylase *in vitro* [5], was tested. We discuss potential mechanisms explaining our data.

Methods

[6RS]-5-H¹³CO-H₄folate was obtained from Schricks Laboratories (Jona, Switzerland). 10-H¹³CO-H₂folate was prepared by air oxidation of [6RS]-10-H¹³CO-H₄folate [5]. 10-HCO-H₄folate and 10-HCO-H₂folate are unstable to oxidation and the fully oxidized form, 10-formylfolic acid (10-H¹³CO-folate), is formed in this process; thus, the maximum possible purity of 10-H¹³CO-H₂folate was ~93% in the freshly prepared compound [5]. Human recombinant H₂folate reductase (DHFR) was a gift from Dr. Vivian Cody at Hauptman-Woodward Medical Research Institute (Buffalo, NY). All other chemicals were from Sigma (St Louis, MO).

After the approval by our Institutional Review Board for Human Use, a healthy adult male (with a 677CC 5,10-methylenetetrahydrofolate reductase genotype) took independent oral doses of [6RS]-5-H¹³CO-H₄folate (25 and 50 mg) or 10-H¹³CO-H₂folate (~23 mg, prepared from 25 mg [6RS]-5-H¹³CO-H₄folate) in 3 separate studies with a 3-month interval between doses. After each dose, he collected urine at every void for 96 h and recorded the time and volume. He maintained his regular life style during urine collection including diets and exercise [2].

The ¹³C-enrichment at the C₂ and C₈ positions of uric acid were measured by an LC/MS/MS method, and calculation of % ¹³C-enrichment was made [2,4]. Briefly, the area-under-the-curve (AUC) of the extracted ion chromatograms (XIC) were determined for: (a) *m/z* 167 → 124 (uric acid containing only ¹²C, ¹H, ¹⁴N and ¹⁶O); (b) *m/z* 168 → 124 (¹³C at C₂); (c) *m/z* 168 → 125 (¹³C at C₈); and (d) *m/z* 169 → 125 (¹³C at both C₂ and C₈). The % ¹³C at C₂ was [b ÷ (a + d)] × 100 and that at C₈ was [c ÷ (a + d)] × 100. Enrichment is the % ¹³C after the ¹³C-labelled folate minus the % ¹³C in baseline experiments, where no dose was given [2].

The natural abundance of the isotopomers of uric acid fragments with C₂ and C₈ was measured in 3-day baseline experiments that were performed twice with a 4-month interval. Data from two baseline experiments were subtracted from each other to measure errors in the method. This resulted in both positive and negative enrichment values, suggesting inherent experimental variations, although it is obvious that the theoretically correct value is zero [2].

The means of % ^{13}C -enrichment in 26 samples were 0.01% ($\pm 0.11\%$, SD) and 0.02% ($\pm 0.08\%$) for C_2 and C_8 , respectively, and they were not significantly different from zero ($p > 0.05$). Considering the above means and the limit of detection of % ^{13}C -enrichment of 0.01, a significant mean % enrichment of ≥ 0.03 would be considered real. Using time-of-day matched baseline values, % ^{13}C -enrichment at C_2 and C_8 after an oral dose of ^{13}C -labeled folates for each void was calculated [2]. The Wilcoxon paired-sample test was used to detect means significantly greater than zero and the runs-above-and-below-the-mean test was used to detect rhythmicity in the data [2]. Values are expressed as mean $\pm$ SD where appropriate.

DHFR activities with folic acid (pteroylglutamic acid), 10-HCO-folate, and 10-HCO- H_2 folate as substrates were assayed by incubating at protein concentration of 2 - 12 $\mu\text{g/mL}$ with 50 mM potassium phosphate (pH 7.4), 50 μM NADPH and 1 mM ascorbate at 23°C . The $\Delta\epsilon$'s at 340 nm of $1.8 \times 10^4/\text{M} \times \text{cm}$ for folic acid and 10-HCO-folate and $1.1 \times 10^4/\text{M} \times \text{cm}$ for 10-HCO- H_2 folate were used to monitor the reduction [5].

Results

The means of % ^{13}C -enrichment in all 3 studies were greater at the C_2 than C_8 position (Table 1). As the dose of [6RS]-5- $\text{H}^{13}\text{CO-H}_4$ folate increased, % ^{13}C -enrichment at C_2 increased from 0.19 to 0.51%, suggesting a dose dependency. The mean C_2/C_8 ratios of ^{13}C -enrichment increased ~ 2 -fold ($0.19/0.08 = 2.4$ [25-mg dose] compared to $0.51/0.11 = 4.6$ [50-mg dose]). The mean % ^{13}C -enrichment at C_8 after [6RS]-5- $\text{H}^{13}\text{CO-H}_4$ folate (25 and 50 mg) was significantly above zero, whereas that after 10- $\text{H}^{13}\text{CO-H}_2$ folate was not (Table 1). The ^{13}C -enrichment at both positions was not correlated with void volumes, uric acid levels or total uric acid in each void.

The ^{13}C -enrichment at C_2 and C_8 of uric acid are shown in Fig. 1 for 32 voids after the oral dose of ^{13}C -folates. After either a 25- or 50-mg dose of [6RS]-5- $\text{H}^{13}\text{CO-H}_4$ folate, % ^{13}C -enrichment at C_2 was generally greater than that at C_8 . When the dose of 10- $\text{H}^{13}\text{CO-H}_2$ folate was used, the highest % ^{13}C -enrichment at C_2 was observed with a clear circadian rhythm, whereas the ^{13}C -enrichment at C_8 with zero or negative values occurred more frequently (18 of 32 voids) than after the doses of [6RS]-5- $\text{H}^{13}\text{CO-H}_4$ folate (Fig. 1). Peaks and valleys of the plot of % ^{13}C -enrichment at C_2 coincided to those at C_8 after a 50-mg dose [6RS]-5- $\text{H}^{13}\text{CO-H}_4$ folate; however, this pattern was not apparent after a 25-mg dose of this folate. Highest enrichment at C_2 occurred at voids in the evening and early morning.

The C_2/C_8 enrichment ratio in each void increased as the enrichment at C_2 increased. For example, the median C_2/C_8 ratio was 1.0 ($n = 18$), when the enrichment of C_2 was $< 0.15\%$ after the 25-mg dose of [6RS]-5- $\text{H}^{13}\text{CO-H}_4$ folate. On the other hand, when the enrichment of C_2 was $> 0.39\%$, the C_2/C_8 ratios were higher with a median of 9.8 ($n = 7$). Similarly, in the study with a 50-mg dose of [6RS]-5- $\text{H}^{13}\text{CO-H}_4$ folate, the median C_2/C_8 ratio was 2.2 ($n = 9$), when % ^{13}C -enrichment of C_2 was $< 0.32\%$. In contrast, the median C_2/C_8 ratios was 6.6 ($n = 18$), when the enrichment of C_2 was $> 0.40\%$. After the dose of 10- $\text{H}^{13}\text{CO-H}_2$ folate, only a few positive C_2/C_8 ratios could be calculated, and we observed a ratio as high as 98. The ratios included in these calculations represented only the positive ones.

The V_{max} of DHFR with 10-HCO- H_2 folate as a substrate was 787 ± 65 (SD) pmol product/ μg protein/min and was constant over substrate concentrations 1.3 - 54 μM . Thus, the K_m could not be accurately estimated; however, it was probably $\leq 0.2 \mu\text{M}$. The V_{max} and K_m of DHFR with folic acid were 386 ± 16 pmol/ μg protein/min and $1.61 \pm 0.26 \mu\text{M}$, respectively. Therefore, it appears that 10-HCO- H_2 folate was a better substrate than folic acid for DHFR. DHFR activity with 50 μM 10-HCO-folate was only 3.0 ± 4.2 pmol/ μg protein/min.

Discussion

Both C₂ and C₈ positions of the purine ring were ¹³C-enriched after oral doses of [6*RS*]-5-H¹³CO-H₄folate, and C₂ was exclusively enriched by 10-H¹³CO-H₂folate. To our knowledge, this is the first demonstration of the absorption and metabolism of 10-HCO-H₂folate in man *in vivo*. 10-HCO-H₄folate produced from 5-HCO-H₄folate is theoretically used equally by GAR and AICAR transformylases (i.e., C₂/C₈ = 1.0); however, 10-H¹³CO-H₄folate was apparently better used by AICAR transformylase (C₂/C₈ > 1.0) in this study. Since only one healthy adult male was studied, a note of caution is necessary in extrapolating the data presented here to all human subjects, such as children and pregnant women as well as patients with certain diseases such as cancer.

To explain our data, we offer the following three non-mutually exclusive mechanisms: I) substrate channeling; II) AICAR transformylase in erythrocytes; and III) conversion of [6*RS*]-5-H¹³CO-H₄folate to 10-H¹³CO-H₂folate and its utilization by AICAR transformylase. However, there may be other explanations.

I) Benkovic and colleagues [6,7] reported that chicken-liver GAR transformylase, but only a small amount of AICAR transformylase, forms an enzyme complex with the trifunctional enzyme (5,10-methylenetetrahydrofolate dehydrogenase, 5,10-CH=H₄folate cyclohydrolase and 10-HCO-H₄folate synthetase) and serine hydroxymethyltransferase. This complex could channel one-C units (eg. from serine) to form 10-H¹²CO-H₄folate, which is then furnished to the active site of GAR transformylase. This channeling would dilute 10-H¹³CO-H₄folate coming from outside this complex and result in a reduction of ¹³C-enrichment at C₈. In contrast, less dilution occurs at C₂, since only a small amount of AICAR transformylase is in this complex [6,7]. Such a dilution of ¹³C at C₈ could be overcome by a higher dose of [6*RS*]-5-H¹³CO-H₄folate (50 mg), and there was a small increase in ¹³C-enrichment at C₈ with this higher dose (Table 1).

II) AICAR transformylase, but not GAR transformylase, is present in human erythrocytes, which may have utilized a substantial amount of 10-H¹³CO-H₄folate to form IMP, resulting in preferential ¹³C-enrichment at C₂ [8,9]. It is known that human erythrocytes metabolize the base and riboside of AICAR to IMP in the presence of 10-HCO-H₄folate [8,9]. Thus, the final steps of PNB *de novo* in humans may be carried out more extensively by erythrocytes than by the liver. AICAR (as the riboside) may be exported from mammalian liver to erythrocytes for the completion of PNB *de novo*, and only a small amount of 10-H¹³CO-H₄folate may have escaped utilization by erythrocytes to reach hepatic GAR transformylase [2]. This explanation is plausible, since AICAR appears to be metabolically inactive in rat hepatocytes [10]; therefore, it can be used as an activator of AMP-activated protein kinase [11].

III) In this study, we used [6*RS*]-5-H¹³CO-H₄folate, of which only [6*S*]-5-H¹³CO-H₄folate (active isomer, 50% of total [6*RS*]-5-HCO-H₄folate) is enzymatically converted to [6*R*]-10-H¹³CO-H₄folate (active isomer). This created a pool of [6*R*]-10-H¹³CO-H₄folate, which can be utilized by both GAR and AICAR transformylases. In addition, [6*R*]-5-H¹³CO-H₄folate potentially created, non-enzymatically, a pool of [6*S*]-10-H¹³CO-H₄folate (inactive isomer), which can be oxidized to 10-H¹³CO-H₂folate [5,12]. We have previously presented data suggesting the *in vivo* chemical conversion of [6*R*]-5-HCO-H₄folate to 10-HCO-H₂folate in humans [3]. This 10-H¹³CO-H₂folate is utilized by AICAR transformylase (not GAR transformylase), and ¹³C-enriches only C₂ [5]. This would explain why the total % ¹³C-enrichment by a 50-mg dose of [6*RS*]-5-H¹³CO-H₄folate (0.62% = 0.51 + 0.11) is 4.1 times the total from a 23-mg dose of 10-H¹³CO-H₂folate (0.15% = 0.14 + 0.01). Since only 50% of [6*RS*]-5-H¹³CO-H₄folate is the active isomer, in theory, bioactivity of both [6*S*]-5-H¹³CO-H₄folate and 10-H¹³CO-H₂folate should be about equal. Therefore, a 4.1-fold difference

suggests that much of the inactive [6R]-5-H¹³CO-H₄folate was converted to 10-H¹³CO-H₂folate.

We found little ¹³C-enrichment at C₈ by the 10-H¹³CO-H₂folate dose. Enrichment at C₈ would require that 10-H¹³CO-H₂folate be reduced to [6R]-10-H¹³CO-H₄folate by DHFR for subsequent utilization by GAR transformylase. Although this reduction occurs *in vitro*, it is likely *in vivo* that most of 10-H¹³CO-H₂folate is utilized by AICAR transformylase before it is reduced by DHFR. Therefore, we hypothesize that the following cyclic metabolic process for the introduction of C into C₂ during PNB *de novo* takes place:

The oxidation of 10-HCO-H₄folate to 10-HCO-H₂folate (Step 2) has been demonstrated *in vitro* [12]. This metabolic cycle is similar to folate coenzyme utilization by thymidylate synthase where DHFR is also required (Step 4) [1].

This is the first study using ¹³C-formylfolates to study PNB *de novo* in a human. Our non-invasive method can be used to investigate the role of formylfolates in this metabolic pathway.

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Fig.1.

The % ^{13}C enrichment at C_2 and C_8 of uric acid in each void after oral doses of $[6\text{RS}]\text{-5-H}^{13}\text{CO-H}_4\text{folate}$ and $10\text{-H}^{13}\text{CO-H}_2\text{folate}$. Bars represent ^{13}C -enrichment at C_2 (solid) and C_8 (open) in voids collected for 96 hours after dosing. The x-axis is void number which represents time after dosing; however, the intervals between voids were different. Therefore, we did not show specify time of the void.

Mean % ¹³C enrichment (± SD) at the C₂ or C₈ positions of urinary uric acid and the enrichment ratios (C₂/C₈) after doses of [6RS]-5-H¹³CO-H₄folate and 10-H¹³CO-H₂folate¹

Table 1

Compound	Dose (mg)	Number of voids	¹³ C-enrichment at C ₂	¹³ C-enrichment at C ₈	¹³ C-enrichment ratio C ₂ /C ₈
[6RS]-5-H ¹³ CO-H ₄ folate	25	32	0.19 ± 0.31	0.08 ± 0.02	2.4
[6RS]-5-H ¹³ CO-H ₄ folate	50	32	0.51 ± 0.21 ²	0.11 ± 0.09	4.6
10-H ¹³ CO-H ₂ folate	23 ³	32	0.14 ± 0.27	0.01 ± 0.06 ⁴	14

¹ All means were significantly greater than zero unless otherwise indicated.

² Significantly higher than that after the 25-mg dose of [6RS]-5-H¹³CO-H₄folate and the dose of 10-H¹³CO-H₂folate (Student *t*-test).

³ Based on 93% purity of the compound.

⁴ The value was not significantly different from zero.