Hydroxyproline Metabolism and Oxalate Synthesis in Primary Hyperoxaluria

Sonia Fargue,1 Dawn S. Milliner,2 John Knight,1 Julie B. Olson,2 W. Todd Lowther,3 and Ross P. Holmes1

1Department of Urology, University of Alabama at Birmingham, Birmingham, Alabama; 2Mayo Clinic Hyperoxaluria Center, Division of Nephrology and Hypertension, Rochester, Minnesota; and 3Center for Structural Biology, Department of Biochemistry, Wake Forest School of Medicine, Winston-Salem, North Carolina

ABSTRACT

Background Endogenous oxalate synthesis contributes to calcium oxalate stone disease and is markedly increased in the inherited primary hyperoxaluria (PH) disorders. The incomplete knowledge regarding oxalate synthesis complicates discovery of new treatments. Hydroxyproline (Hyp) metabolism results in the formation of oxalate and glycolate. However, the relative contribution of Hyp metabolism to endogenous oxalate and glycolate synthesis is not known.

Methods To define this contribution, we performed primed, continuous, intravenous infusions of the stable isotope [15N,13C5]-Hyp in nine healthy subjects and 19 individuals with PH and quantified the levels of urinary 13C2-oxalate and 13C2-glycolate formed using ion chromatography coupled to mass detection.

Results The total urinary oxalate-to-creatinine ratio during the infusion was 73.1, 70.8, 47.0, and 10.6 mg oxalate/g creatinine in subjects with PH1, PH2, and PH3 and controls, respectively. Hyp metabolism accounted for 12.8, 32.9, and 14.8 mg oxalate/g creatinine in subjects with PH1, PH2, and PH3, respectively, compared with 1.6 mg oxalate/g creatinine in controls. The contribution of Hyp to urinary oxalate was 15% in controls and 18%, 47%, and 33% in subjects with PH1, PH2, and PH3, respectively. The contribution of Hyp to urinary glycolate was 57% in controls, 30% in subjects with PH1, and 13% in subjects with PH2 or PH3.

Conclusions Hyp metabolism differs among PH types and is a major source of oxalate synthesis in individuals with PH2 and PH3. In patients with PH1, who have the highest urinary excretion of oxalate, the major sources of oxalate remain to be identified.


Endogenous oxalate synthesis makes an important contribution to calcium oxalate stone disease and is the main cause for the pathologies that develop in the rare, recessive, inherited primary hyperoxaluria (PH) disorders.1 Three genetic forms of PH have been identified, associated with mutations in AGXT for PH1, GRHPR for PH2, and HOGA1 for PH3 (Figure 1, Supplemental Figure 1). PH1 and PH2 both result from deficiencies in enzymes that metabolize glyoxylate to other compounds. In PH1, the deficiency in the peroxisomal, pyridoxine-dependent, liver-specific enzyme, alanine:glyoxylate aminotransferase (AGT1), prevents the conversion of glyoxylate to glycine.2,3 In PH2, the deficiency in glyoxylate reductase/hydroxypyruvate reductase

Received April 7, 2017. Accepted March 6, 2018.

Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Ross P. Holmes, Department of Urology, University of Alabama at Birmingham, 720 20th Street, South, Kaul 816, Birmingham, AL 35294. Email: rholmes@uab.edu

Copyright © 2018 by the American Society of Nephrology
(GRHPR; also known as GR) prevents the conversion of glyoxylate to glycolate in mitochondria and cytosol.4–6 PH3 results from mutations in the mitochondrial enzyme 4-hydroxy-2-oxoglutarate aldolase (HOGA).7,8 HOGA catalyzes the last step of hydroxyproline (Hyp) catabolism converting 4-hydroxy-2-oxoglutarate (HOG) to glyoxylate and pyruvate. It has been shown that HOG inhibits GR activity.9 Thus, PH3 has also been proposed to be a disorder of glyoxylate metabolism. Outside of supportive measures, there are few therapeutic options for individuals with PH apart from the approximately 30% of patients with PH1 who respond fully or partially to therapeutic doses of the cofactor for AGT, pyridoxine.10 Liver/kidney transplantation is often the only treatment option for those with severe disease. Thus, development of new treatments is an important focus of current PH research.

Our understanding of how oxalate is synthesized in PH is incomplete. Glyoxylate is thought to be the major precursor of endogenous oxalate synthesis in PH.1 Sources of glyoxylate synthesis include Hyp, glycin, glyoxal, and glycolate11–11 (Figure 1). However, the relative contributions of these compounds to glyoxylate and oxalate synthesis are unknown in healthy individuals and notably in the different PH types. In addition to the increased oxalate, there is often an increased urinary excretion of glycolate in PH1, which is the most frequent of all PH types and carries the most severe clinical outcomes.12 Dietary studies in mice, rats, pigs, and humans have indicated that Hyp ingestion results in increased endogenous oxalate synthesis and urinary oxalate excretion.13–17 Although it cannot be synthesized de novo, Hyp is a normal blood and tissue constituent with collagen turnover as its major source. Hyp is produced by a post-translational hydroxylation of proline residues principally in collagen.18 Our recent studies in normal mice infused with [15N,13C5]-Hyp indicated that, under the experimental conditions used, Hyp contributed approximately 20% of the oxalate excreted in urine.19 These results have recently been confirmed in HYPDH null mice (Prodh2 gene) that cannot metabolize Hyp and excreted approximately 20% less oxalate than wild-type mice on a low-oxalate diet (J. Knight, W.T. Lowther, and R.P. Holmes, unpublished results).

The objective of this study was to determine the contribution of Hyp metabolism to urinary oxalate and glycolate in healthy subjects and individuals with PH, using continuous, intravenous infusions of [15N,13C5]-Hyp and measurements of 13C-labeled metabolites of Hyp in plasma and urine. This information will be of value in the identification of novel therapeutic targets for treatment of patients with PH.

**Figure 1.** Metabolism of Hyp and glyoxylate in hepatocytes. Oxalate synthesis by lactate dehydrogenase (LDH) is stimulated in three types of patients with PH by defects in either AGT (PH1, "**"), glyoxylate reductase (GR; PH2, "***"), or HOGA (PH3, "****") (see Supplemental Figure 1 for detailed changes to metabolism that occur for each PH type). Hyp is converted enzymatically to HOG in three steps. The action of HOGA results in the formation of glyoxylate and pyruvate. Mutations in HOGA lead to a buildup of HOG and its reduced form, DHG. Importantly, the glyoxylate and glycolate are interconverted by the action of GR and glycolate oxidase (GO). D-amino oxidase (DAO) is also a potential source of glyoxylate. Whereas GR is both cytosolic and mitochondrial and expressed ubiquitously, AGT and GO are peroxisomal and liver specific. The factors that influence movement of metabolites between different subcellular compartments are not known, but previous work and this study support exchange of glyoxylate metabolites between mitochondrial and peroxisomal pathways, as well as a level of redundancy and/or plasticity in these two pathways.21

**Significance Statement**

The primary hyperoxalurias (PH) are a group of rare, inherited disorders characterized by kidney stone formation and severe kidney damage resulting from excessive endogenous oxalate synthesis. Limited treatment options are available for PH. Hydroxyproline metabolism, known to contribute to oxalate synthesis, is a potential target for oxalate reduction therapy. Using a stable isotope of hydroxyproline, the contribution to urinary oxalate excretion was measured in healthy and PH subjects. Our results show that hydroxyproline accounts for up to half of the endogenous oxalate synthesis in PH2 and PH3, more than twice that of healthy subjects. In PH1, surprisingly, the bulk of the oxalate synthesized in PH1 was not derived from hydroxyproline and its source remains to be determined.

**METHODS**

**Subjects**

A total of nine healthy subjects (5 males [M]/4 females [F]) were studied at Wake Forest School of Medicine and the University of Alabama at Birmingham. Nineteen patients with PH (12M/7F) attended the Hyperoxaluria Center at the Mayo Clinic (Rochester, MN) for treatment. The inclusion criteria for patients were age 15–85 years; confirmed eGFR > 50 ml/min per 1.73 m² using plasma creatinine20; a

**Figure 1.** Metabolism of Hyp and glyoxylate in hepatocytes. Oxalate synthesis by lactate dehydrogenase (LDH) is stimulated in three types of patients with PH by defects in either AGT (PH1, "**"), glyoxylate reductase (GR; PH2, "***"), or HOGA (PH3, "****") (see Supplemental Figure 1 for detailed changes to metabolism that occur for each PH type). Hyp is converted enzymatically to HOG in three steps. The action of HOGA results in the formation of glyoxylate and pyruvate. Mutations in HOGA lead to a buildup of HOG and its reduced form, DHG. Importantly, the glyoxylate and glycolate are interconverted by the action of GR and glycolate oxidase (GO). D-amino oxidase (DAO) is also a potential source of glyoxylate. Whereas GR is both cytosolic and mitochondrial and expressed ubiquitously, AGT and GO are peroxisomal and liver specific. The factors that influence movement of metabolites between different subcellular compartments are not known, but previous work and this study support exchange of glyoxylate metabolites between mitochondrial and peroxisomal pathways, as well as a level of redundancy and/or plasticity in these two pathways.21
genetically confirmed diagnosis of PH1, PH2, or PH3; and no history of transplantation for the PH group. Six of the PH1 subjects had at least one missense mutation and were treated with pyridoxine, which was maintained during the study. Four of these patients with PH1 carried a single allele with a known B6-responsive mutation (p.Gly170Arg or p.Phe152Ile). For control subjects, a normal serum and urine profile and good health assessed by their medical history was required. To eliminate the confounding effect of dietary oxalate, the intake of dietary oxalate and calcium was controlled for the infusion. For 3 days preceding the infusions, but not for baseline assessment, the study subjects consumed a low-oxalate diet (50–100 mg of oxalate) containing 16% protein, 30% fat, and 54% carbohydrate; 200 mg of ascorbate; and 1000–1200 mg of calcium, in order to normalize their metabolism. Foods high in Hyp were avoided. The study was approved by the Institutional Review Boards of the respective participating institutions.

\[^{15}\text{N},^{13}\text{C}_3\]-Hyp Intravenous Infusion

The infusions were performed in the postabsorptive state. After a 10-hour overnight fast, and waking at 6:00 AM, subjects emptied their bladders and drank 750 ml of water. Subjects were admitted to the Clinical Research Unit at 7:00 AM. Infusions were initiated at 8:00 AM, with a priming dose of \[^{15}\text{N},^{13}\text{C}_3\]-Hyp (0.75 \mu\text{mol/kg}) and D3-leucine (2 \mu\text{mol/kg}) administered over a 5-minute period in 0.9% saline. This was followed immediately by a constant infusion of 0.75 and 2 \mu\text{mol/h per kg} for \[^{15}\text{N},^{13}\text{C}_3\]-Hyp and D3-leucine, respectively, for 6 hours. Separate catheters were used for the infusion (antecubital vein) and blood collection (superficial hand vein of the opposite arm). A 1-hour preinfusion urine sample was collected. Blood and urine samples were collected every half-hour and every hour, respectively, during the infusion. Subjects drank 250 ml water per hour during the infusion (infusion scheme in Figure 2).

Chemicals

Reagent grade chemicals were obtained from either Sigma-Aldrich Chemicals (St Louis, MO) or Fisher Scientific (Pittsburgh, PA). \[^{15}\text{N},^{13}\text{C}_3\]-Hyp (the nitrogen and all five carbons were isotopically labeled) was obtained from Almac following a custom synthesis (Craigavon, United Kingdom),19,21 and D3-leucine from Cambridge Isotopes (Andover, MA).

Sample Preparation

Aliquots of urine samples from the infusion were acidified to a concentration of 50 mM HCl (final pH<2) before freezing to prevent possible oxalate crystallization that could occur with cold storage or oxalogenesis associated with alkanization. Nonacidified urine was frozen for the measurement of other urinary parameters. All plasma and urine samples from patients with PH were shipped frozen and stored at −80°C. For plasma analyses by ion chromatography coupled with mass spectrometry (IC/MS), samples were filtered through Spin-X UF centrifugal filters (Corning, NY) with a 10-kD nominal molecular mass limit, and the ultrafiltrate was acidified by the addition of 2 M HCl to a final concentration of 60 mM (final pH<2). Centrifugal filters were washed with 0.01 M HCl before sample filtration to remove any contaminating trace organic acids trapped in the filter device.

Analytic Methods

Urinary creatinine was measured in nonacidified urine on an EasyRA chemical analyzer. Plasma Hyp concentration was measured by high-pressure liquid chromatography coupled with UV detection using AccQ-Fluor reagents (Waters, Milford, MA) according to the manufacturer’s instructions. The mole percent enrichment of \[^{15}\text{N},^{13}\text{C}_3\]-Hyp and D3-Leucine in plasma was measured by gas chromatography–mass spectrometry by Metabolic Solutions (Nashua, NH). Ion chromatography using an AS15, 2×150 mm, anion exchange column coupled with negative-mode electrospray mass spectrometry (IC/MS) (Thermo Fisher Scientific Inc., Waltham, MA) was used to measure glycolate and mole percent enrichment with \(^{13}\text{C}_2\)-glycolate in acidified urines and plasma, as previously described.20 Total oxalate and mole percent enrichment with \(^{13}\text{C}_2\)-oxalate were determined in acidified urine by an IC/MS method recently developed in our laboratory. An IonPac 5 \mu m AS22, 2×150 mm, anion exchange column with guard was operated at 0.20 ml/min and room temperature using 20 mM ammonium carbonate as the mobile phase. The use of ammonium carbonate for IC renders the detection of conductivity impossible, but its volatility makes it compatible with the mass detector under these conditions. The mass detector (MSQ; Thermo Fisher Scientific Inc., Waltham, MA) was operated in ESI negative mode, needle voltage 0.5V, cone voltage 30V,
and an MS source temperature of 400°C. The column eluent was mixed with 50% acetonitrile at 0.4 ml/min using a zero-dead volume mixing tee before entry into the mass spectrometer. Selected-ion monitoring (SIM) at the following mass/charge ratios, $^{13}\text{C}_2$-oxalate (SIM 89) and $^{13}\text{C}_2$-glyoxylate (SIM 91), was used to determine total oxalate and the mole percent enrichment of $^{13}$C$_2$-oxalate in the samples. Percent mole $^{13}\text{C}_2$-oxalate enrichment was determined using standards with known amounts of $^{13}\text{C}_2$-oxalate in the range 0%-50% mole percent enrichment. Total oxalate levels in samples were determined by standard isotope dilution analysis using $^{13}\text{C}_2$-oxalate. Urine isomers of HOG (SIM 161, 166), dihydroxyglutarate (DHG) (SIM 163, 168), and glycerate (SIM 105) were determined by IC/MS, using an AS11-HC 4 μm anion exchange hydroxide column operated at 0.38 ml/min and 30°C. The limits of detection for total and $^{13}$C mole percent enrichment (above natural abundance) for each analyte were the following: total glycolate (0.02 μM), $^{13}$C$_2$-glycolate (0.8% mole enrichment), total oxalate (0.1 μM), $^{13}$C$_2$-oxalate (0.2% mole enrichment), total glycerate (0.02 μM), and total HOG (2 μM). Because pure standards for DHG are not available, 2-hydroxyglutarate was used as a standard to estimate DHG concentration.

**Calculations**

Calculations are on the basis of $^{13}$C-labeled samples obtained after equilibration of the isotopic tracer ($[^{15}\text{N},^{13}\text{C}_5]$-Hyp steady state). Detailed methods and the rationale for tracer use are described in the Supplemental Material.

The whole-body turnover rate or flux of Hyp (Q$_{\text{Hyp}}$) was calculated as described in Equation 1.\textsuperscript{23–25}

$$Q = \frac{i}{(E_i/E_{\text{Hyp}}) - 1}$$  \hspace{1cm} (1)

where “i” is the $[^{15}\text{N},^{13}\text{C}_5]$-Hyp infusion rate in micromoles per kilogram per hour and $E_i/E_{\text{Hyp}}$ is the ratio of isotopic enrichment of the $[^{15}\text{N},^{13}\text{C}_5]$-Hyp infusion rate ($E_i$, 94%) and the mole percent enrichment of the total Hyp plasma pool with $[^{15}\text{N},^{13}\text{C}_5]$-Hyp ($E_{\text{Hyp}}$) (referred to as “enrichment” in the text).

Because the $[^{15}\text{N},^{13}\text{C}_5]$-Hyp tracer is also the precursor to the labeled products $^{13}\text{C}_2$-oxalate and $^{13}\text{C}_2$-glycolate, the production of oxalate and glycolate from Hyp metabolism was quantified using the following method:

The overall contribution of Hyp catabolism to urinary oxalate ($U_{\text{Ox}}$) and glycolate ($U_{\text{Glc}}$) excretion is determined by the mole percent enrichment of urine with $^{13}\text{C}_2$-oxalate and $^{13}\text{C}_2$-glycolate corrected for the fraction of labeled $[^{15}\text{N},^{13}\text{C}_5]$-Hyp that is circulating in the plasma (Equation 2).\textsuperscript{19,23}

$$\text{Hyp Contribution} = \frac{(E_{\text{Uox}} + E_{\text{UGlc}})}{E_{\text{Hyp}}} \times 100$$  \hspace{1cm} (2)

where $E_{\text{Uox}}$ and $E_{\text{UGlc}}$ are the mole percent enrichment of urine with $^{13}\text{C}_2$-oxalate and $^{13}\text{C}_2$-glycolate, respectively (referred to as “enrichment” in the text).

The amount of urinary oxalate and glycolate derived from Hyp metabolism is estimated as the absolute amount synthesized, defined as the percent contribution of Hyp to urinary metabolite (calculated by Equation 2), multiplied by the size of the relevant pool (the urine excretion of that metabolite), expressed as ratio to creatinine excretion.

**Statistical Analyses**

Statistical analyses were conducted in GraphPad Prism v. 7. Comparison of data between groups was analyzed with one-way ANOVA followed by Tukey’s test for multiple comparisons if $P<0.05$. A paired t test was used to compare repeated measures between different time points, such as preinfusion (T0) and postinfusion (T360). Equilibration over time was analyzed by repeated-measures one-way ANOVA analysis followed by Tukey’s post hoc test. In addition to parametric tests, nonparametric analyses (Kruskall–Wallis and Wilcoxon tests) were also performed and confirmed the conclusion drawn. Data are expressed as mean±SEM, unless otherwise indicated. The criterion for statistical significance was $P<0.05$; all tests were two-sided.

**RESULTS**

Demographic and clinical characteristics of the control and PH groups are shown in Table 1; individual subject data and PH genotype information are presented in Supplemental Tables 1 and 2. No significant differences between the subject groups were observed for body mass index and GFR. On the basis of preinfusion (T0) urine collections on an oxalate-controlled diet (Table 1), urinary oxalate excretion was higher in PH groups compared with normal subjects. PH1 subjects showed significant individual variation that appeared related to their pyridoxine responsiveness. Urinary glycolate excretion was significantly different between the groups; highest in the PH1 and lowest in the PH3 group, with some PH1 subjects in the normal range as expected.\textsuperscript{10} Urinary glycerate was highest in patients with PH2, whereas HOG and DHG were highest in patients with PH3, as expected given their specific PH molecular defects (Figure 1, Supplemental Figure 1). Mean
plasma Hyp levels, before infusion (T0), were significantly lower in individuals with PH compared with control subjects.

The kinetics of Hyp metabolism and the formation of two of its products, glycolate and oxalate, were studied using stable isotope tracer methodology, similar to methods established for the study of other amino acids.19,23,24 A primed, continuous infusion of the stable isotope tracer, [15N, 13C5]-Hyp, was performed intravenously and serial samples were collected until equilibration of the tracer in plasma. The amount of the [15N, 13C5]-Hyp tracer at steady state relative to the total Hyp concentration (defined as the isotopic enrichment) yields information on the daily turnover of Hyp. The enrichment of 13C2-oxalate and 13C2-glycolate in urine yields information on how much oxalate and glycolate in urine is derived from the metabolism of Hyp. The latter is defined as the contribution, using calculations described in the Methods (see Supplemental Figure 2, Supplemental Material).

Six-hour infusions of [15N, 13C5]-Hyp and D3-leucine, a common infusion control, were performed in all subjects (Figure 2). The enrichment of the total plasma Hyp pool with [15N, 13C5]-Hyp reached steady state in 5–6 hours (Supplemental Figure 3), and was higher in PH1, PH2, and PH3 subjects (32%, 40%, and 28% versus 19% in controls, respectively; P<0.001). This may be related to the lower

Table 1. Baseline characteristics and urinary and plasma values before and after [15N, 13C5]-Hyp infusion at 0.75 μmol/h per kg

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls</th>
<th>PH1</th>
<th>PH2</th>
<th>PH3</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>9</td>
<td>7</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>29.3±2.0</td>
<td>25.7±2.1</td>
<td>[PH2]</td>
<td>51.5±11.1</td>
<td>37±6.6</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>6/3</td>
<td>4/3</td>
<td>0/4</td>
<td>8/0</td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.2±1.2</td>
<td>24.6±1.1</td>
<td></td>
<td>26.2±3.3</td>
<td>28±2.6</td>
</tr>
<tr>
<td>GFR, ml/min per 1.73 m²</td>
<td>105±6.5</td>
<td>112.1±4.0</td>
<td></td>
<td>129±17.7</td>
<td>97.5±9.7</td>
</tr>
<tr>
<td>B6 treatment</td>
<td>0</td>
<td>6 of 7</td>
<td>0</td>
<td></td>
<td>2 of 8</td>
</tr>
<tr>
<td>24-h urine profile</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine oxalate, mg/g creat</td>
<td>18.9±2.2</td>
<td>[PH2]</td>
<td>63.2±23.1</td>
<td>88.9±19.1</td>
<td>52.4±6.6</td>
</tr>
<tr>
<td>Urine glycolate, μM</td>
<td>4.5±0.3</td>
<td>26.9±10.2</td>
<td>[C, PH3]</td>
<td>7.4±1.1</td>
<td>a</td>
</tr>
<tr>
<td>Plasma glycolate, μM</td>
<td>10.9±0.4</td>
<td>[PH1,2,3]</td>
<td>6.1±0.5</td>
<td>8.4±0.8</td>
<td>a</td>
</tr>
<tr>
<td>Plasma Hyp, μM</td>
<td>13.2±1.5</td>
<td>72.3±24.6</td>
<td>69.5±12.2</td>
<td>43.4±6.1</td>
<td>0.07</td>
</tr>
<tr>
<td>Urine glycolate, mg/g creat</td>
<td>23.9±5.7</td>
<td>63.4±23.1</td>
<td>[PH3]</td>
<td>33.0±6.8</td>
<td>9.4±2.0</td>
</tr>
<tr>
<td>End of infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma oxalate, μM</td>
<td>3.4±0.5</td>
<td>5.7±1.5</td>
<td>3.6±0.2</td>
<td>4.3±0.4</td>
<td>4.7±0.7</td>
</tr>
<tr>
<td>Plasma glycolate, μM</td>
<td>3.9±0.3</td>
<td>25.5±9.6</td>
<td>[C, PH3]</td>
<td>4.4±0.5</td>
<td>3.0±0.3</td>
</tr>
<tr>
<td>Plasma Hyp, μM</td>
<td>10.1±0.5</td>
<td>8.3±0.6</td>
<td>9.0±1.1</td>
<td>9.5±0.4</td>
<td>0.14</td>
</tr>
<tr>
<td>Urine oxalate, mg/g creat</td>
<td>10.4±1.4</td>
<td>[PH1,3]</td>
<td>65.2±20.4</td>
<td>57.0±8.8</td>
<td>49.0±4.0</td>
</tr>
<tr>
<td>Urine glycolate, mg/g creat</td>
<td>30.4±7.3</td>
<td>54.1±13.6</td>
<td>[PH3]</td>
<td>16.7±2.8</td>
<td>13.4±2.6</td>
</tr>
<tr>
<td>Urine glycerate, mg/g creat</td>
<td>4.6±1.3</td>
<td>2.1±0.4</td>
<td>248±84.8</td>
<td>[C, PH1,3]</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>Urine HOG, mg/g creat</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>25.4±1.6</td>
<td>12.8±3.1</td>
</tr>
<tr>
<td>Average excretion during infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine oxalate, mg/g creat</td>
<td>10.6±0.6</td>
<td>[PH1]</td>
<td>73.1±27.9</td>
<td>70.8±18.8</td>
<td>47.0±4.0</td>
</tr>
<tr>
<td>Urine glycolate, mg/g creat</td>
<td>23.3±4.0</td>
<td>[PH1]</td>
<td>59.6±16.7</td>
<td>[PH3]</td>
<td>25.4±1.6</td>
</tr>
</tbody>
</table>

Each subject provided two 24-h urine collections of which the mean was used for the 24-h urine profile determinations. Urine values before the start of the infusion (also called T0, “before infusion”) and after the infusion of [15N, 13C5]-Hyp infusion (also called T360, “end of infusion”) were determined in hourly collections, started the hour before that time. The average excretion during infusion was calculated as the mean of all seven hourly values during the infusion and was used as total urine excretions for the calculation of contributions. Urine excretions are expressed as a ratio to creatinine excretion. Results expressed as mean±SEM, *P values shown for comparisons between groups. Statistical analyses were done to compare data between groups and to compare data before and after infusion (see Methods). Significant differences between subject groups according to Tukey’s post hoc test are specified [different from subject group] directly to the right of the means columns analyzed, [C]: different from the control group. M/F, male/female; BMI, body mass index; creat, creatinine.

*Significant difference between T0 and T360 infusion time points P<0.05.
baseline plasma Hyp levels seen in those groups (Table 1). In contrast, the enrichment of the D3-leucine was not different between the groups and reached steady state within 2 hours (results not shown), similar to previous reports by us and others.23,24 To determine whether the increase in plasma Hyp with [15N,13C5]-Hyp infusion (0.75 μmol/h per kg) altered metabolism, we infused a subset of four normal subjects with a lower rate of [15N,13C5]-Hyp (0.15 μmol/h per kg). This resulted in enriching the plasma Hyp pool by 3.7%, a value approximately five-fold lower than that achieved with the higher infusion rate.

There was no significant difference in the Hyp turnover rate between infusion rates of 0.15 and 0.75 μmol/h per kg (P=0.15). However, the lower infusion dose compromised our detection of 13C2-oxalate, because the levels obtained were close to the limit of quantitation. Therefore, the Hyp turnover rate was determined in all subjects at the 0.75 μmol/h per kg infusion rate. There were no statistical differences between pre- and postinfusion samples for urinary oxalate, glycerate, HOG, and DHG, and plasma oxalate at the 0.75 μmol/h per kg infusion rate (Table 1). Altogether, these data support that the Hyp infusion protocol we applied did not alter the normal synthesis of the metabolites under study, as appropriate for a metabolic tracer.

The levels of the urine metabolites of [15N,13C3]-Hyp, 13C2-oxalate and 13C2-glycolate, did not reach steady state within the 6-hour-long infusion (Supplemental Figure 4). These observations are consistent with a delay in the time taken for metabolites to equilibrate within intracellular pools, as we have previously observed with glycine infusions.23

The excretion of total oxalate, glycolate, 13C2-oxalate, and 13C2-glycolate levels were determined in hourly urinary collections (Table 1). These measurements and those in the final collection (T360 minutes) permitted the calculation of the amount of urinary oxalate derived from the catabolism of Hyp; 12.8, 32.9, and 14.8 mg oxalate/g creatinine in PH1, PH2, and PH3, respectively, compared with 1.6 mg oxalate/g creatinine in controls (Figure 3A). The isotopic enrichment of urinary oxalate with 13C2-oxalate and plasma Hyp with [15N,13C3]-Hyp at the end of the infusion were used to determine the percent contribution of Hyp catabolism to total urinary oxalate excretion (see Supplemental Material). The proportional contribution of Hyp catabolism (Figure 4) to total urinary oxalate excretion was similar in PH1 subjects and controls (18% versus 15%, respectively), but was higher in PH2 (47%) and PH3 (33%) (P<0.05). The contribution in PH3 subjects, however, showed variable results, with three of the eight PH3 subjects in the range of controls (Figure 4A).

The amounts of glycolate and 13C2-glycolate derived from Hyp catabolism are shown in Figure 3B. Hyp contributed a mean 57% of total urinary glycolate excretion in normal subjects (Figure 4B). This contribution was lower in all PH types compared with controls. Low amounts of 13C2-glycolate were detected in the urine of all patients with PH2, indicating that a pathway of glyoxylate to glycolate exists in the absence of GR. In addition to oxalate, urinary 13C2-glycolate was detected in four of eight PH3 subjects, suggesting that glyoxylate can be synthesized from HOG (Figure 1), and that this process is variable in patients with PH3. 13C2-HOG (2.3 mg/g creatinine) and 13C2-DHG (0.4 mg/g creatinine) were detected in the last urine collection from six of eight and eight of eight patients with PH3, respectively (Table 1). These latter data further confirm that Hyp metabolism in individuals with PH3 includes conversion of HOG to DHG.

The mean Hyp turnover rate was 3.1 μmol/h per kg in healthy subjects
Figure 5. Overview of the endogenous synthesis and excretion of oxalate and glycolate in healthy subjects and patients with PH. The estimated amount of urinary oxalate and glycolate excreted in 24-hour urine that is derived from Hyp metabolism is expressed as mg/g creatinine (see Figures 3 and 4 for details). The relative contribution of oxalate and glycolate derived from Hyp to the urinary oxalate and glycolate pools is expressed as percentage of total urinary excretion.

(Supplemental Figure 5) compared with 1.5 and 1.1 μmol/h per kg, respectively, in PH1 and PH2 subjects. In PH3 the mean Hyp turnover rate was also lower (2 μmol/h per kg), but the distribution of values was broader and demonstrated an age-related decrease (Supplemental Figure 6).

DISCUSSION

In treating PH, reduction in endogenous oxalate production is at the heart of therapeutic strategies. The search for effective strategies is hampered by a lack of knowledge of pathways of endogenous oxalate synthesis (Figure 1). Hyp metabolism results in oxalate formation, but its contribution to urinary oxalate excretion in humans was not previously known. In this study we used constant infusions of a stable isotope of Hyp to demonstrate that Hyp metabolism is an important source of urinary oxalate excretion in all forms of PH, with the greatest proportional contribution in PH2 and PH3. In PH1, which is the most severe form of PH, sources other than Hyp appear to play a prominent role in endogenous oxalate and glycolate synthesis and remain to be clarified.

Hyp Metabolism

We observed that Hyp metabolism contributes 15% of the oxalate excreted in urine of normal individuals. Prior studies with $^{13}$C$_2$-glycine infusion suggest that glycine metabolism contributes up to 5%. Dietary oxalate accounts for 40% of total urinary oxalate excretion,$^{23}$ with the remaining 60% derived from endogenous oxalate synthesis. Thus, in healthy subjects, Hyp and glycine combined account for approximately one third of endogenous oxalate synthesis. Other sources that contribute to oxalate synthesis could include glycolate not derived from Hyp metabolism (e.g., glycolate derived from phosphoglycolate formed during DNA repair$^{22}$), glyoxal,$^{12}$ and ascorbic acid.$^{26}$

In PH2 subjects Hyp was responsible for almost half of the endogenous oxalate produced, the highest contribution of Hyp among all groups (Figures 4 and 5). This result demonstrates that GR, deficient in PH2, plays an important role in limiting the metabolism of Hyp-derived glyoxylate to oxalate (Supplemental Figure 1). Mitochondrial GR activity may be particularly important in this glyoxylate detoxification process, and its role is likely to be accentuated in the renal proximal tubule, which lacks AGT but expresses enzymes of the Hyp catabolism pathway. The low but detectable amounts of $^{13}$C$_2$-glycolate observed in individuals with PH2 (Supplemental Figure 4, Table 1) suggest that another enzyme, such as lactate dehydrogenase, could catalyze the conversion of glyoxylate to glycolate.$^1$ The contribution of Hyp to oxalate production observed in PH2 subjects raises the possibility that a therapy blocking HYPDH activity could be efficacious in reducing urinary oxalate in this group. HYPDH deficiency (MIM #237000) in humans does not cause clinical symptoms$^{27,28}$ and HYPDH null mice lack symptoms while having an approximately 20% reduced urinary oxalate excretion (J. Knight, W.T. Lowther, and R.P. Holmes, unpublished results). These observations support targeting liver and kidney HYPDH as an approach for oxalate reduction therapy in PH2.

In PH3 subjects, Hyp catabolism was also an important source of urinary oxalate. The contribution of Hyp to urinary oxalate was on average higher (33%, Figure 4) than control subjects but quite variable and in some cases even similar to that of healthy subjects, despite the HOGA deficiency (Figure 4).$^9$ This heterogeneity extended to urinary levels of HOG, DHG, and Hyp contribution to urinary glycolate. This suggests that additional enzymes may be capable of metabolizing HOG, to either glyoxylate or DHG, with varying degrees between individuals. Several clinical reports mention a decline of symptoms with age in some patients with PH3. These observations, in conjunction with the decrease in Hyp turnover rate with age in PH3 subjects (Supplemental Figure 6), hint at the possibility of age-related changes or disease adaptations in
Hyp metabolism. The pathophysiology of PH3 and the mechanisms leading to the increased oxalate synthesis have been the subject of debate. We have previously shown that HOG inhibits recombinant GR activity and have hypothesized that this results in increased endogenous oxalate synthesis in PH3 (Supplemental Figure 1). The trend toward lower total glycolate excretion in PH3 subjects compared with healthy individuals and other PH types supports this mechanism. On the other hand, the formation of urinary $^{13}$C$_2$-glycolate and $^{13}$C$_2$-oxalate in PH3 subjects also supports the hypothesis that an alternative aldolase activity converts HOG to glyoxylate. Strategies that block HYPD activity could also benefit patients with PH3, given that HOGA, the enzyme affected by mutations in PH3, catalyzes a reaction downstream in the Hyp catabolic pathway. However, the heterogeneity of the production of HOG and its metabolites in patients with PH3 highlights the need for a greater understanding of the metabolic pathways.

PH1 subjects exhibited a greater synthesis of oxalate, total and derived from Hyp, compared with controls (Figure 3A). Interestingly, the proportional contribution of Hyp catabolism to urinary oxalate was similar to that of healthy subjects (Figures 4 and 5). Other pathways of endogenous oxalate synthesis seem to play an important role in this disorder. Our observations underscore that the metabolic deficiencies leading to PH1 are quite different from those of PH2 and PH3 and appear to rely on peroxisomal rather than mitochondrial pathways. The AGT deficiency in PH1 establishes an open cycle of glycolate-glyoxylate interconversion in the liver (Supplemental Figure 1B); i.e., oxidation of glycolate to glyoxylate by peroxisomal GO and reduction of glyoxylate to glycolate by cytosolic GR. This cycling most likely plays an important role in the pathophysiology of PH1, due to the hydrogen peroxide produced with each cycle. Recent studies by our group and others have shown that reducing GO activity with siRNA in the Agxt-deficient mouse results in a significant decrease in urinary oxalate excretion, further supporting that glycylate metabolism is a major source of oxalate in PH1, independent of Hyp catabolism. Subsequent work with rats and monkeys further supported GO as a viable siRNA knock down target for oxalate reduction therapy, and clinical trials in patients with PH1 using GO siRNA are being conducted. This therapeutic approach, however, will increase glycolate levels. Preliminary studies in our laboratory show that isolated human proximal tubules, which lack GO, can convert glycolate to oxalate (S. Fargue, J. Knight, and R.P. Holmes, unpublished results), which could result in increased renal oxalate synthesis in the event of increased glycolate production with siRNA treatment.

**Hyp Turnover**

The bulk of the Hyp that enters the extracellular fluid compartment is derived from collagen turnover with bone being a major source, exceeding that of skin, muscle, and other tissues. It is possible that some of the Hyp-rich body pools exchange slowly with the extracellular Hyp pool in addition to an unknown intersubject variability in these processes. This could in part explain the extended time required for infused $^{15}$N,$^{13}$C$_3$-Hyp to approach steady state. In our study, the mean total body turnover rate of Hyp in normal individuals was $3.1 \mu$mol/h per kg, which is equivalent to a turnover of 660 mg Hyp/d or 4.7 g collagen/d for a 70-kg individual. This is almost twice the rates published by Kiivirikko, which were on the basis of the urinary Hyp excretions of a 12-year-old female and a 31-year-old male with hydroxyprolinemia due to a presumed HYPD deficiency. Reasons for the prior underestimation of Hyp turnover rate may include inadequate methodology for estimating Hyp at that time and possible Hyp degradation through other metabolic pathways in these individuals. The reasons for the lower Hyp turnover rates (Supplemental Figure 5) and lower total plasma Hyp seen in patients with PH in our study are unclear. One possible explanation would be that a lower turnover of collagen takes place in response to the increased oxalate production that occurs in PH, thus partially limiting glyoxylate production upstream of the enzymatic deficiencies. Importantly, despite lower Hyp turnover from bone and other soft tissues and lower blood levels of Hyp in PH, the metabolism of Hyp accounts for quantitatively larger amounts of oxalate excreted in the urine in patients with all three forms of PH (Figure 5).

**Limitations and Future Perspective**

The number of subjects enrolled in each group was small due to the rarity of the disease; the complexity of infusions; and the time, expense, and travel involved, particularly for patients. Genotypic and age differences in individuals with PH, unavoidable in such a study, may have contributed to variations in their responses. The true contribution of Hyp to oxalate and glycolate production may be underestimated in our study, because steady states of the $^{15}$N,$^{13}$C$_3$-Hyp metabolites, $^{13}$C$_2$-oxalate and $^{13}$C$_2$-glycolate, were not reached at the end of the infusion and the contribution of dietary oxalate absorption to urinary oxalate may change during the course of the fast. Alternative study designs could be on the basis of longer infusions but metabolism may be significantly altered with an extended fast, and studies conducted in the fed state could create complexities limiting their interpretation. Future studies will be needed to elucidate the remaining sources of endogenous oxalate synthesis, which are prominent in PH1, and the role that the glycolate to glyoxylate cycling plays in oxalate synthesis.

**ACKNOWLEDGMENTS**

We thank Zhixin Wang, Nicole Holderman, and Alex Dowell for their excellent technical assistance. We greatly appreciate the participation of the subjects with primary hyperoxaluria and the healthy subjects and the cooperation of the Clinical Research Units at Wake Forest School of Medicine, UAB (supported by National Institutes of Health [NIH] grant TR001417), and the Mayo Clinic (supported by NIH grant TR000135). This work was presented as an abstract at the 2016 annual meeting of the American Society of Nephrology Kidney Week, Chicago, IL, November 15–20, 2016. ClinicalTrials.gov identifier NCT02038543.
R.P.H., J.K., W.T.L., and D.S.M. designed the study; S.F., J.K., and J. B.O. carried out experiments and patient recruitment; S.F. and J.K. analyzed the data; S.F., J.K., R.P.H., W.T.L., and D.S.M. drafted and revised the paper; all authors approved the final version of the manuscript.

DISCLOSURES

None.

REFERENCES


5. Cramer SD, Ferree PM, Lin K, Milliner DS, Holmes RP: The gene encoding hydroxyproyruvate reductase (GRHPR) is mutated in patients with primary hyperoxaluria type II. Hum Mol Genet 8: 2063–2069, 1999


34. Laitinen O: The metabolism of collagen and its hormonal control in the rat with special emphasis on the interactions of collagen and calcium in the bones. Acta Endocrinol (Copenh) 56(Suppl) 120: 1–86, 1967


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2017040390/-/DCSupplemental.