γ-Glutamyl Hydrolase, Not Glutamate Carboxypeptidase II, Hydrolyzes Dietary Folate in Rat Small Intestine\textsuperscript{1,2}

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Abstract

Dietary polyglutamyl folates are hydrolyzed to monoglutamyl folic acid derivatives prior to intestinal transport. In humans and pigs, the reaction occurs at pH 6.5 at the jejunal brush border membrane by folic acid hydrolase and is encoded by the glutamate carboxypeptidase II (GCPII) gene. Intracellular folic acid hydrolase with an optimal pH of 4.5 is encoded by the γ-glutamyl hydrolase (γ-GH) gene and predominates in rats. We determined the respective roles of GCPII and γ-GH in dietary folate hydrolysis in rats. Duodenal, jejunal, ileal mucosa, pancreas, and duodenal luminal fluid were collected from 10 Sprague-Dawley rats that had not been food deprived. Folate hydrolase was assayed at pH 4.5 and 6.5 with and without parahydroxymercuribenzoate (pHMB), an inhibitor of intracellular folic acid hydrolase. Folate hydrolase activity occurred at pH 4.5 in all tissues, was significantly inhibited by the addition of pHMB at both pH 4.5 and 6.5, and was virtually absent from brush border fractions at pH 6.5. The highest activity was in the postprandial duodenal luminal fluid at pH 4.5. Rat-specific primers for GCPII and γ-GH were used to detect mRNA expression in pancreas, jejunal mucosa, and liver. GCPII expression was detected only in the liver, whereas γ-GH was expressed in all 3 tissues. These results suggest that the hydrolysis of polyglutamyl folates in rats requires the intracellular folic acid hydrolase that is expressed by pancreatic γ-GH, in contrast to GCPII that is expressed in the jejunal mucosal brush border in pigs and humans. γ-GH folic acid hydrolase is abundant in rat postprandial pancreatic secretions and appears to hydrolyze dietary folates in the intestinal lumen prior to intestinal absorption. J. Nutr. 137: 1149–1153, 2007.

Introduction

Folate is an essential water soluble vitamin that is required for normal growth and development. Humans and other mammals cannot synthesize folate and must acquire it from the diet. Dietary folates are a mixture of monoglutamyl and polyglutamyl forms that are hydrolyzed to the monoglutamyl form prior to intestinal transport. Both the intestinal hydrolysis of polyglutamyl folates and the intestinal transport across the jejunal brush border membrane are essential in the assimilation of dietary folates.

Glutamate carboxypeptidase II (GCPII)\textsuperscript{3} is expressed in pig and human small intestine as folic acid hydrolase, which is the enzyme predominantly involved in the hydrolysis of dietary polyglutamyl folates. GCPII is also expressed in rat brain as N-acetylated α-linked acidic dipeptidase (1) and in human prostate as prostate-specific membrane antigen (PSMA) (2). Although all 3 proteins are expressed by GCPII, only folic acid hydrolase (intestinal GCPII) is involved in dietary polyglutamyl folate hydrolysis. Intestinal GCPII is a zinc-activated endopeptidase that is highly expressed in pig and human jejunal brush border membranes with an optimal pH of 6.5 (3,4) and sequentially cleaves terminal γ-linked glutamate residues from dietary polyglutamyl folates prior to the uptake of the monoglutamyl folate products across the same membrane (3).

Wang et al. (5) compared folate hydrolase activities among various species and found high levels of jejunal brush border membrane activities in pigs and humans but negligible amounts in rats and monkeys (5). In a separate study, rat jejunal brush border membrane folic acid hydrolase activity was found to be upregulated by folate deficiency, but the levels of activity under all experimental conditions were very low compared with other species (6).

Originally identified as an intracellular intestinal folate hydrolase (4), γ-glutamyl hydrolase (γ-GH) expresses a soluble endopeptidase glycoprotein that cleaves γ-linked glutamate residues from polyglutamyl folates, is optimally active over a pH range of 4.5–6.0, and has been identified in pig, human, rat and monkey intestine as well as human liver, kidney, placenta, colon and brain (7,8,4,5). In contrast to intestinal GCPII, γ-GH is localized in the lysosomal fraction of enterocytes and hepatocytes (4) and is found in serum, bile, and pancreatic secretions (8,9).

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\textsuperscript{1} Supported by the Jastro-Shields fellowship award at the University of California, Davis.

\textsuperscript{2} Author disclosure: T. B. Shafizadeh, no conflicts of interest; and C. H. Halsted, no conflicts of interest.

\textsuperscript{3} Abbreviations used: γ-GH, γ-glutamyl hydrolase; GCPII, glutamate carboxypeptidase II; p-HMB, parahydroxymercuribenzoate.

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Although both enzymes hydrolyze polyglutamyl folates, the genes for GCPII and γ-GH have distinct sequences and are expressed in different locations. As a type II membrane protein, intestinal GCPII is highly glycosylated, a modification that allows the enzyme to be targeted and anchored to the jejunal brush border membrane and is necessary for its hydrolytic activity (10,11). From this location, GCPII hydrolyzes dietary folates entering the intestinal lumen prior to intestinal absorption. Conversely, γ-GH is involved in intracellular polyglutamyl folate hydrolysis, which probably allows for the generation of intracellular monoglutamyl folate, enabling its exit from the cell and entry into the circulation (8,12,4).

The mechanism for the hydrolysis of polyglutamyl folate in rats has not been confirmed. Early studies by Kesavan and Noronha (13) showed that incubation of polyglutamyl folates with rat luminal contents yielded monoglutamyl folates, in contrast to a study in which incubation with human luminal aspirates did not hydrolyze a mixture of labeled polyglutamyl folate (14). Kesavan and Noronha (9) also identified rat luminal folate hydrolase activity of pancreatic origin that could be induced by the ingestion of dietary polyglutamyl folates but not by the ingestion of synthetic monoglutamyl folates. In these experiments, rat pancreatic folate hydrolase activity showed a 2-fold increase, and proximal intestinal fluid showed an 8- to 9-fold increase, following the ingestion of polyglutamyl folates, whereas no increase occurred after the consumption of monoglutamyl folates. Additionally, pancreatectomized rats showed impaired hydrolysis and uptake of ingested dietary polyglutamyl folates, evident by the decreased rise in serum folate, whereas the rise in serum folate after ingesting monoglutamyl folates was unaffected by pancreatectomy (9).

The objective of the present study was to re-evaluate the role of the pancreas in the initial digestion of dietary folate in rats and to determine the respective roles of γ-GH and intestinal GCPII in polyglutamyl folate hydrolysis in this species.

Methods

Chemicals. RNAlater solution was purchased from Ambion. Complete Protease Inhibitor Cocktail tablets were purchased from Roche Applied Science. [14C]PteGlucose was a gift from Dr. Carlos Krumdieck (University of Alabama). Bio-Rad Protein Assay was obtained from Bio-Rad Laboratories. Trizol Reagent and the First Strand Synthesis kit were purchased from Invitrogen. All PCR reagents were obtained from Qiagen. Primers were synthesized by the Molecular Structure Facility at the University of California, Davis. All other chemicals were obtained from various commercial sources.

Animals and tissue collection. Male Sprague-Dawley rats (n = 10) were purchased from Charles River Laboratories and were fed a diet of Purina Rodent Chow 5008 (Ralston Purina) ad libitum (15). This study complied with the Guide for the Use and Care of Laboratory Animals at the University of California, Davis, which is accredited by the Animal Association for the Accreditation of Laboratory Care, and protocols for rat maintenance were approved by the UC Davis Animal Care and Use Committee. Immediately following asphyxiation in a CO2 chamber, the pancreas, duodenal intestinal contents, and the entire small intestine distal to the pylorus were removed from each rat. Luminal duodenal contents were collected by clamping and cutting the distal end of the duodenum and manually expressing the intestinal contents into a microcentrifuge tube. Segments of the small intestine were identified and collected as follows: –7.5 cm each of duodenum (between the pyloric sphincter and the ligament of Trietz), jejunum (immediately distal to the ligament of Trietz), ileum (immediately proximal to the ileo-cecal junction) were excised, and mucosal scrapings from each segment were collected using glass slides. The tissue samples were wrapped in foil and immediately frozen in dry ice. In addition, 20 mg of pancreas and each intestinal segment were placed in RNAlater solution to stabilize RNA for future isolation. All tissue samples were maintained at –80°C until analysis.

Tissue preparation. Frozen intestinal mucosa and pancreas were homogenized in tris-mannitol buffer containing protease inhibitor cocktail using a Polytron homogenizer (Brinkman Instruments). Brush border membranes were isolated from mucosal scrapings by the calcium precipitation method (16), and purity was determined by measuring the enrichment of alkaline phosphatase activity. In all samples, brush border membrane fractions showed a 6- to 10-fold higher alkaline phosphatase specific activity compared with total homogenate (data not shown). The final membrane pellets were resuspended in 150 μL of homogenization buffer containing the protease inhibitor cocktail. Luminal duodenal contents were centrifuged at 32,000 × g for 20 min to separate undigested food particles. Luminal duodenal fluid was removed, and the remaining pellet was discarded.

Enzyme activity. Based on initial kinetic studies conducted in our laboratory (12), the folate hydrolase activities of γ-GH and intestinal GCPII were measured in tissue homogenates, intestinal brush border membranes, and luminal duodenal fluid by the methods of Krumdieck and Baugh (17) using 12 μmol/L [14C]PteGlucose as substrate. The 14C radiolabel is located on the terminal glutamate residue of the polyglutamyl folate so that both the exopeptidase activity of intestinal GCPII and the endopeptidase activity of γ-GH can liberate the labeled glutamate and the rate of folate hydrolysis can be measured after charcoal precipitation of unreacted folate. The 3,3-dimethylglutarate buffer was adjusted to pH 4.5 and pH 6.5 to measure the different folate hydrolase activities, respectively. Parahydroxymercuribenzoate (p-HMB, 0.17 mmol/L) was added to the reaction mixture to inhibit the folate hydrolase activity of γ-GH for select samples. p-HMB has been shown in prior studies to completely inhibit intracellular folate hydrolase activity, whereas brush border membrane folate hydrolase activity is unaffected (12,4,5). Samples were incubated with radiolabeled substrate for 40 min at 37°C before terminating the reaction with 10% trichloroacetic acid. Two percent charcoal in 0.1 mol/L acetic acid was then added to bind uncleaved substrate. After centrifugation at 2000 × g for 10 min, supernatants containing radiolabeled product were collected and measured using a liquid scintillation counter. Folate hydrolase activity in luminal duodenal fluid was measured over a pH range of 3.0–8.0 by adjusting the pH of the 3,3-dimethylglutarate buffer with 1 mol/L NaOH. Protein concentration was measured by spectrophotometry using Bio-Rad reagent at 595 nm. Folate hydrolase–specific activity was expressed as pmol cleaved substrate mg protein–1 min–1.

RT-PCR. Total RNA from all tissue samples except luminal duodenal fluid was isolated using the Trizol reagent method (18). Intestinal cDNA was prepared by reverse transcription using Invitrogen First Strand Synthesis kit. The quality of cDNA was analyzed by PCR using 18S primers. Rat-specific primers for γ-GH and GCPII were designed using Primer Express software (Applied Biosystems). The γ-GH primers included forward (GGCTGTCCGGATCCTATGAG) and reverse (CCGGAAACATTCTGCTCTGCT) and GCPII primers included forward (GGTAAGTCCATACGATGGGTTG) and reverse (GTGCTCCACTCTGAGGTC). The melting temperature for both primer sets was 58°C, and PCR were carried out for 36 cycles. Two μL of PCR product were loaded onto a 2.5% agarose gel containing ethidium bromide and was electrophoresed at 100 v for 25 min. Bands corresponding to γ-GH and GCPII transcripts were visualized using UV detection.

Statistical analysis. ANOVA was used to compare folate hydrolase activity among tissues at each pH and in the presence or absence of p-HMB (α = 0.05). Student’s paired t test was used to compare folate hydrolase activity within each tissue at each pH. Student’s t test for unequal variance was used to compare activity in luminal fluid at each pH without p-HMB. Activity assays were performed in triplicate. Values are presented as the mean ± SEM (n = 10), and differences were considered significant at P ≤ 0.01.
Results

Folate hydrolase activity. In an initial pilot study, total intestinal mucosal homogenate, brush border membrane, cytosol and microsomal fractions of rat proximal jejunum were assayed at pH 4.5 and 6.5. Folate hydrolase activity was 1.6 to 24 times greater at pH 4.5 than at pH 6.5 in jejunal homogenates, cytosol, and microsomes. Jejunal cytosolic folate hydrolase activity peaked at pH 4.5 with minimal activity detected at pH 6.5, consistent with the activity of γ-GH. The highest activity was found in the cytosolic fraction at pH 4.5, whereas negligible activity was detected at either pH in jejunal brush border membranes. The results of the pilot study were the basis for the experiments conducted in the present study.

Folate hydrolase activity in duodenal, jejunal, and ileal total mucosal homogenates was measured at pH 4.5 and 6.5 alone and with the addition of 0.17 mmol/L pHMB, an inhibitor of intracellular folate hydrolase activity that has no effect on membrane folate hydrolase activity (12). In this experiment, folate hydrolase activity was detected at pH 4.5 in all segments of rat small intestine mucosal homogenate, but activities were significantly lower at pH 6.5 (Table 1). The addition of pHMB blocked all folate hydrolase activity at both pH 4.5 and 6.5 in all segments of the intestine, except there was a minimal jejunal activity at pH 6.5 in the presence of pHMB.

Previous experiments in pigs and humans showed that intestinal folate hydrolase activity is located primarily on the jejunal brush border membrane where intestinal GCPII activity is optimal at pH 6.5 and is not inhibited by pHMB (13,20,5). However, except for the low activity at pH 6.5 in the jejunal homogenate noted above, no activity was detected in rat small intestinal mucosal homogenate after pHMB treatment, consistent with the lack of GCPII expression. To further confirm this finding, the same activity measurements were performed on duodenal, jejunal, and ileal brush border membrane vesicles (Table 2). Although folate hydrolase activity was detected in all segments of rat intestinal brush border membrane at pH 4.5, the addition of pHMB eliminated all detectable activity in this fraction at both pH 4.5 and pH 6.5. These findings agree with those described for the total homogenate (Table 1) and support the hypothesis that GCPII is not present in rat small intestine. The presence of folate hydrolase activity at both pH 4.5 and 6.5 in rat intestinal brush border membrane and its total inhibition with pHMB suggest possible intracellular contamination of the brush border membrane vesicle fractions during preparation.

Based on earlier studies suggesting a role for pancreatic folate hydrolase in rats (8,9), we next measured folate hydrolase activity at pH 4.5 and 6.5 with and without pHMB in the pancreas total homogenate and in the postprandial fluid expressed from the duodenal lumen (Table 3). Folate hydrolase activity in luminal fluid at pH 4.5 was >4.5-fold higher than that in all segments of rat small intestine (Table 1) or in the pancreas at both pH 4.5 and 6.5. The addition of pHMB reduced pH 4.5 folate hydrolase activity in luminal fluid by 99% and in pancreas by 90% and reduced pH 6.5 activity in pancreas by 99% and luminal fluid activity at pH 6.5 by 71%. These findings are consistent with the intracellular origin of pancreatic and luminal fluid folate hydrolase activity.

γ-GH and GCPII mRNA expression. To further establish the identities of the enzymes involved in folate hydrolysis in various tissues in rats, RT-PCR of jejunal mucosa and pancreas cDNA was performed using rat-specific primers for γ-GH and GCPII (Fig. 1). RT-PCR was utilized to determine relative differences among tissues rather than to quantify absolute levels of mRNA transcripts. Rat liver cDNA was used as a positive control for both genes. γ-GH transcripts were detected in all 3 rat tissues with the highest expression in the pancreas. In contrast, GCPII transcripts were detected at low levels in rat liver, but not in pancreas or jejunum, indicating a lack of GCP II expression in these tissues.

Discussion

In pigs and humans, dietary polyglutamyl folates are hydrolyzed by jejunal brush border folate hydrolase to their monoglutamyl forms prior to transport across this membrane. Unlike prior findings in pigs and humans (5), intestinal GCPII activity, as defined by pH optimum 6.5 and lack of pHMB inhibition, was not detected in rat jejunal brush border membrane in the present study (Table 2). Previously, Said et al. (6) reported folate hydrolase activity in rat intestinal brush border membrane. However,

**TABLE 1** Folate hydrolase specific activity in rat small intestine mucosal homogenate with and without pHMB

<table>
<thead>
<tr>
<th>Tissue</th>
<th>pH</th>
<th>Alone</th>
<th>With pHMB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmol</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- mg protein(^{-1}) - min(^{-1})</td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>4.5</td>
<td>44.5 ± 12.7</td>
<td>— (^2)</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td>6.3 ± 3.7*</td>
<td>—</td>
</tr>
<tr>
<td>Jejunum</td>
<td>4.5</td>
<td>24.1 ± 3.9</td>
<td>—</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td>3.4 ± 0.7*</td>
<td>0.5 ± 0.9</td>
</tr>
<tr>
<td>Ileum</td>
<td>4.5</td>
<td>28.4 ± 5.3</td>
<td>—</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td>1.9 ± 1.1*</td>
<td>—</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 10. *Different from pH 4.5.
2 Activity was considered undetectable when the activity value in the blank sample was higher than the sample in question (i.e. a negative activity value).

**TABLE 2** Folate hydrolase activity in rat small intestine brush border membrane vesicles

<table>
<thead>
<tr>
<th>Tissue</th>
<th>pH</th>
<th>Alone</th>
<th>With pHMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>4.5</td>
<td>46.3 ± 3.5</td>
<td>— (^2)</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td>— *</td>
<td>—</td>
</tr>
<tr>
<td>Jejunum</td>
<td>4.5</td>
<td>2.0 ± 0.9</td>
<td>—</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td>2.5 ± 0.8</td>
<td>—</td>
</tr>
<tr>
<td>Ileum</td>
<td>4.5</td>
<td>9.0 ± 2.7</td>
<td>—</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td>0.3 ± 4.1*</td>
<td>—</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 10. *Different from pH 4.5.
2 Activity was considered undetectable when the sample blank had a higher activity value than the sample in question (i.e. a negative activity value).

**TABLE 3** Folate hydrolase activity in rat pancreas and duodenal luminal fluid

<table>
<thead>
<tr>
<th>Tissue</th>
<th>pH</th>
<th>Alone</th>
<th>With pHMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreas</td>
<td>4.5</td>
<td>57.0 ± 9.0</td>
<td>5.6 ± 4.3</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td>63.8 ± 9.5</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Luminal Fluid</td>
<td>4.5</td>
<td>348.0 ± 134.8</td>
<td>4.0 ± 3.1</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td>15.4 ± 2.6*</td>
<td>4.5 ± 1.8</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 10. *Different from pH 4.5.
Previous studies showed that folate hydrolase is expressed in rat pancreas and secreted into the proximal intestinal lumen (9,5). Although pancreatic secretion of folate hydrolase activity was previously reported in pigs (23), it is thought to play a minor role in dietary polyglutamyl folate hydrolysis compared with that of jejunal GCPII in humans and pigs (3,19). To date, the roles of γ-GH and intestinal GCPII in dietary folate hydrolysis by rats have not previously been determined. The results of the present study confirm that γ-GH is expressed in the pancreas with the probable origin of its content in the intestinal lumen and, rather than GCPII, appears to be involved in the hydrolysis of dietary folate in rats. Additionally, our study demonstrates contrasting mechanisms of intestinal folate hydrolysis in rats, humans, and pigs, confirming prior studies that suggest the use of the rat model for human dietary folate digestion and absorption may not be appropriate (5,24).

**Literature Cited**


