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Starvation alters the activity and mRNA level of glutaminase and glutamine synthetase in the rat intestine

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The metabolism of glutamine, the main respiratory fuel of enterocytes, is governed by the activity of glutaminase and glutamine synthetase. Because starvation induces intestinal atrophy, it might alter the rate of intestinal glutamine utilization. This study examined the effect of starvation on the activity, level of mRNA, and distribution of mRNA of glutaminase and glutamine synthetase in the rat intestine. Rats were randomized into groups and were either: (1) fed for 2 days with rat food ad libitum or (2) starved for 2 days. Standardized segments of jejunum and ileum were removed for the estimation of enzyme activity, level of mRNA, and in situ hybridization analysis. The jejunum of the fed rats had a greater activity of both enzymes per centimeter of intestine ($P < 0.01$), a greater glutaminase specific activity (1.97 ± 0.45 vs. 1.09 ± 0.34 μmol/hr/mg protein, $P < 0.01$), and a lower level of glutaminase and glutamine synthetase mRNA. The ileum of the fed rats had a greater activity of glutamine synthetase per centimeter of intestine (162.9 ± 50.6 vs. 91.0 ± 23.1 nmol/hr/cm bowel, $P < 0.01$), a lower level of glutaminase mRNA, and a greater level of glutamine synthetase mRNA. In situ hybridization analysis showed that starvation does not alter the distribution of glutaminase and glutamine synthetase mRNA in the intestinal mucosa. This study confirms that starvation decreases the total intestinal activity per centimeter of both glutaminase and glutamine synthetase. More importantly, the results indicate that the intestine adapts to starvation by accumulating glutaminase mRNA. This process prepares the intestine for a restoration of intake.

Keywords: starvation; intestine; glutaminase; glutamine synthetase mRNA

Introduction

The studies of Windmueller and Spaeth[1] established that glutamine, a five-carbon amino acid, is the main respiratory fuel of enterocytes. It was demonstrated that carbon derived from glutamine contributed 46% of the carbon dioxide released from isolated perfused segments of rat jejunum. In addition to its role as a fuel, glutamine may be important to the intestine because it is also a source of substrates for the synthesis of nucleic acids. Indeed, it has been argued that one reason for the high rate of glutamine degradation in enterocytes and other cells, such as lymphocytes and macrophages, is to ensure regulation of the rate of nucleic acid synthesis.[2] The metabolism of glutamine is governed by the activity of two enzymes: glutaminase and glutamine synthetase. Although it is known that starvation can reduce the activity of intestinal glutaminase and glutamine synthetase,[3,4] it is not known what effect starvation has on the level or distribution of mRNA of these enzymes. The aim of this study was to examine the effect of starvation, for 2 days, on the activity, level of mRNA, and distribution of mRNA of glutaminase and glutamine synthetase in the intestine of rats.

Materials and methods

The experimental protocol was approved by the Royal Perth Hospital Animal Ethics Committee.

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Animals and treatments

Twelve adult male Wistar rats (350–400 g) were allowed 1 week after transport to acclimatize before entry into the experiment. Throughout the period of the study, the rats were kept in communities (two per cage), exposed to constant temperature and a 12-hour light/dark cycle, and given water ad libitum. The animals were allocated to one of the two groups ($n = 6$ per group) under study by using a blocked strategy on the basis of computer-generated pseudo-random numbers. The rats were either: (1) fed for 2 days with Rat and Mouse Cubes (Glen Forrest Stockfeeders, Perth, Western Australia, Australia; fed group) or (2) starved for 2 days (starved group). The Rat and Mouse Cube diet is manufactured from wheat, soybean meal, meat meal, salt, beef fat, canola oil, vitamins, and minerals, and contains 14.5 MJ/kg of digestible energy, 18.9% protein, 5.2% fat, 5% crude fiber, 0.77% calcium, 0.57% phosphorus, and 0.41% salt.

Harvest procedures

At the end of the study period, the rats were anesthetized by an intraperitoneal injection of 50 mg/kg of pentobarbitone sodium. The rats were weighed and 5 mL of blood removed from the inferior vena cava. The blood was placed in tubes containing ethylenediamine-tetraacetic acid (EDTA). The small intestine was excised and the lumen flushed with 5 mL of ice-cold normal saline. The jejunum and ileum were divided into 10 cm, 1 cm, and 0.5 cm long segments. The 0.5 cm long sections were placed in vials filled with Optimal Cutting Temperature Compound (OCT; Finetechnical Co. Ltd., Tokyo, Japan) and the 1 cm long sections were placed in microfuge vials. All vials were snap-frozen in liquid nitrogen and stored at −70°C before analysis. The 10-cm segments of intestine were blotted dry, weighed, and slit down the antimesenteric border. The mucosa was removed from the muscularis by scraping with a glass slide. The sample of intestinal mucosa, which included cells from the intestinal villus and crypts, was weighed and subsampled for the estimation of enzyme activity and concentration of mRNA.

Estimation of glutaminase activity

A modification of the two-step macro-measurement procedure of Curthoys and Lowry[5] was used. Aliquots of isolated enterocytes were homogenized in water (1/3 w/v) using a glass-Teflon homogenizer. The first reaction mixture contained, in a total volume of 40 μL: 20 mM glutamine; 150 mM sodium phosphate; 0.2 mM EDTA; 50 mM Tris(hydroxymethyl)aminomethane-base (Tris-base), pH 8.6; and either 10 μL of jejunal or 20 μL of ileal homogenate. This reaction mixture was incubated at 37°C for 30 min before the addition of 10 μL of 2 M HCl. The second reaction mixture was prepared by adding 1 mL of a solution containing 50 mM Tris-base (pH 8.6), 0.3 mM adenosine diphosphate (ADP), 1 mM nicotinamide adenine dinucleotide phosphate (NADP), 0.03% hydrogen peroxide, and 60 units of glutamate dehydrogenase in glycerol storage solution (Roche Molecular Biochemicals, Sydney, Australia) to the first reaction mixture. This mixture was incubated at 25°C for 30 min and centrifuged at 3,000 g for 10 min, and the absorbance at 340 nm estimated using a spectrophotometer (UltroSpec II, LKB Biochrom Ltd., Science Park, Cambridge, England). The rate of glutamate formation was estimated by extrapolation from a standard curve that ranged from a concentration of 0.00 to 0.22 μM glutamate. Preliminary work demonstrated that the rate of formation of product was linear with respect to both incubation time and amount of homogenate. The protein and DNA content of the homogenates was estimated by the methods of Lowry et al.[6] and Larbarca and Paigen,[7] respectively.

Estimation of glutamine synthetase activity

Glutamine synthetase was extracted from the tissues and enterocytes according to the method of Iqbal and Ottaway.[8] Aliquots of intestinal mucosa were suspended (1/2 w/v) in ice-cold extraction buffer (0.15 M NaCl–5 mM $NaHCO_3$) and stored at −20°C overnight. The next day, the samples were thawed and homogenized using a Teflon homogenizer (Wheaton, Millville, NJ USA). To allow for the complete extraction of glutamine synthetase, the homogenates were stored at 4°C for 2 hr with frequent shaking. They were centrifuged at 48,000 g for 90 min using a J2-MI centrifuge [Beckman Instruments (Australia) Pty. Ltd., Gladesville, NSW, Australia]. The supernatants were placed in a fresh microfuge tube and assayed for glutamine synthetase activity according to the method of Mecke.[9] Each reaction tube contained, in a total volume of 500 μL, 200 mM Tris, pH 7.7; 100 mM hydroxylamine hydrochloride; 10 mM disodium hydrogen arsenate; 1.5 mM ADP; 0.5 mM manganese sulphate; 2 mM glutamine; and either jejunal or ileal homogenate. The tubes were incubated at 37°C for 30 min. At the end of the incubation, 500 μL of ferric chloride reagent (5 g $FeCl_3 \cdot 6H_2O$, 10 g trichloroacetic acid, and 25 mL concentrated HCl dissolved in 300 mL of deionized water) was added to each sample tube. The samples were centrifuged at 240 g for 10 min at 4°C and the absorbances of supernatants measured at 500 nm using a LKB Biochrom Ultrospec II spectrophotometer (LKB Biochrom Ltd.). The rate of γ-glutamylhydroxamate formation was estimated by extrapolation from a standard curve that ranged from a concentration of 0.00 to 1.00 mM γ-glutamylhydroxamate. Preliminary work demonstrated that the rate of formation of product was linear with respect to both incubation time and amount of homogenate. The protein content of the homogenates was estimated by the method of Lowry et al.[6]

Estimation of concentration of mRNA

A semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method[10] was used for the estimation of the concentration of glutaminase mRNA. The 28S ribosomal RNA (28S) was used as a control. The level of ribosomal RNA in the intestine is not affected by either position on the crypt villus axis or starvation for 2 days.[11,12] Total RNA was extracted from the isolated enterocytes using RNAzol B (Bresatec, Adelaide, Australia) according to the manufacturer's instructions. The integrity of the RNA was assessed using denaturing gel electrophoresis and the concentration of the RNA was determined by spectrophotometry (UltroSpec II, LKB Biochrom Ltd.). A RETROscript kit (Ambion Inc., Austin, TX USA) was used to reverse transcribe RNA into complementary DNA (cDNA). A 16 μL premix containing 2 μg RNA, 10 nmol each dNTP, and 100 pmol random decamers in nuclease free water was incubated at 70°C for 3 min. The premix was cooled on ice before the addition of 10 units of Moloney-murine leukemia virus reverse transcriptase, 10 units of placental RNase inhibitor, and 2 μL 10X reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 150 mM $MgCl_2$). The 20 μL RT mixture was incubated at 42°C for 60 min followed by 92°C for 10 min. The resultant cDNA was stored at −20°C.

Preliminary titrations were used to establish the optimum conditions for the estimation of concentration of glutaminase and glutamine synthetase mRNA.[10] The sequences of the glutaminase, glutamine synthetase, and 28S primers were derived from published sequences[13–15] and are presented in *Table 1*. Each 25 μL PCR mixture contained 40 ng cDNA, 0.5 units AmpliTaq Gold Polymerase (Perkin Elmer, Foster City, CA USA), 2.5 nmol each dNTP, and 1.0 μM of either glutaminase or glutamine synthetase primers in reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$). For estimation of the level of glutaminase mRNA, 28S primers were added, to a final concen-

Table 1 Primer sequences and the size of polymerase chain reaction products

	Primer sequences (5′ → 3′)	Size (basepairs)
Glutamine synthetase[15]	ATCTTGCATCGGGTATGCGA AGTAACCCTTCTTCTCCTGG	339
Glutaminase[14]	TGACAAGATGGGCAACAGTG GTTATTCCACCTGTCCTTGG	348
28S Ribosomal RNA[13]	TGAACTATGCTTGGGCAGGG AGCGCCATCCATTTTCAGGG	500

tration of 0.1 μM, to the reaction tubes after 15 cycles of amplification. For estimation of the level of glutamine synthetase mRNA, 28S primers were added, to a final concentration of 0.1 μM, to the reaction tubes after 18 cycles of amplification. This procedure ensured that the exponential phases of amplification of target and internal control overlapped. The reaction proceeded at 94°C for 9 min followed by 28 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min. Five microliters of the PCR mixtures was then subjected to polyacrylamide gel electrophoresis. Gels were stained with Vistra Green (Amersham, Sydney, Australia) and the fluorescence of the products estimated using a Fluorimager SI (Molecular Dynamics, Sunnyvale, CA USA) and the ImageQuant Software Package (Molecular Dynamics). The fluorescence of the glutaminase and glutamine synthetase products was expressed as a ratio of the fluorescence of the 28S products.

In situ hybridization

The glutaminase and glutamine synthetase primers and a PCR Digoxigenin Probe Synthesis Kit (Roche Molecular Biochemicals) were used, according to the manufacturer's instructions, to synthesize digoxigenin-labeled cDNA probes. In situ hybridization was performed using an OmniSlide System (Hybaid Ltd., Ashford, UK) and the Digoxigenin Nucleic Acid Detection Kit (Roche Molecular Biochemicals). All solutions used for in situ hybridization were prepared with either RNase-free reagents and diethylpyrocarbonate treated double deionized water or standard laboratory reagents and double deionized water followed by treatment with diethylpyrocarbonate.

The samples frozen in OCT were sectioned onto silinated glass slides at 6 μm using an Ames Cryostat II (Miles Inc., Torrance, CA USA). Sections were cut perpendicular to the mucosal surface and three sections were placed on each slide. The sections were air-dried for 30 min, delineated using a DAKO Pen (Dako, Carpinteria, CA USA) and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) for 30 min. The sections were then washed for 5 min in PBS containing 0.2% Triton-X followed by two 15-min washes in PBS alone. The sections were treated with 0.1 M HCl for 20 min at room temperature and washed twice in PBS for 15 min. Two hundred microliters of a solution containing 0.1 M Tris-HCl, pH 8.0, 0.05 M EDTA, and 5 μg/mL proteinase K was applied to each section and the slides incubated for 15 min at 37°C. Sections were washed for 5 min in PBS containing 0.1% glycine followed by two 5-min washes in PBS alone. Sections were post-fixed for 15 min in 4% paraformaldehyde and washed twice in PBS for 15 min. Twenty-five microliters of pre-hybridization buffer containing 5X SSC (750 mM NaCl, 0.75 mM sodium citrate, pH 7.2), 5% blocking reagent (Roche Molecular Biochemicals), 50% deionized formamide, 0.1% *N*-laurylsarcosine, and 0.02% sodium dodecyl sulphate was placed on each section and the slides incubated at 37°C for 15 min. Excess pre-hybridization buffer was removed by blotting and 30 μL of pre-hybridization buffer containing the digoxigenin-labeled probe diluted 1 in 8 was placed on each section. Coverslips were placed on sections, and the slides heated at 100°C for 10 min to denature the probe. The sections were incubated overnight at 37°C in a humid chamber. Coverslips were removed and the sections washed once with 2X SSC for 15 min, twice with 1X SSC for 15 min, twice with 0.1X SSC at 37°C for 15 min, and once with Buffer 1 (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5) for 5 min. Two hundred micoliters of blocking reagent (Roche Molecular Biochemicals) was applied to each section and the slides incubated at room temperature for 30 min. Sections were then washed with Buffer 1 for 5 min at 37°C. Two hundred microliters of a solution containing diluted (1 in 500) anti-digoxigenin-alkaline phosphatase Fab fragments conjugate (Roche Molecular Biochemicals) was applied to each section and the sections incubated at 37°C for 60 min. Sections were then washed twice for 15 min with Buffer 1 and equilibrated in Buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 min, and 200 μL of nitro blue tetrazolium/5-bromo-4-chloro-3-indol phosphate solution (Roche Molecular Biochemicals) placed on each section. To allow development of color the slides were incubated in the dark at room temperature. Color development was stopped by applying 100 μL of a solution containing 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA on each section. Finally, sections were washed in water, counter-stained with methyl green, and mounted using Kaiser's mounting medium.

Three controls were used for in situ hybridization analysis: (1) no proteinase K digestion, (2) pretreatment with RNase A (10 μL/mL for 5 min) before denaturation, or (3) use of unlabeled probe.

Plasma and intestinal amino acid content

Samples of rat blood were centrifuged at 3,000 rpm for 10 min and the plasma collected by aspiration. The plasma was ultra-filtered (Ultra-free MC 10,000 NMLL filters, Millipore Corporation, Bedford, MA USA) and stored at −70°C prior to analysis. The 1-cm segments of intestine were removed from the freezer, weighed, and homogenized 1/10 w/v in 0.1 M HCl using a glass-Teflon homogenizer. Homogenates were centrifuged at 1,500 g for 15 min at 4°C and supernatants removed and ultrafiltered (Ultra-free MC 10,000 NMLL filters; Millipore Corporation). The plasma and tissue filtrates were analyzed for glutamine, citrulline, and arginine content according to the Pico Tag Method,[16] using a high performance liquid chromatograph (Waters Corporation, Bedford, MA USA). The Maxima software package (Waters Corporation) was used to identify and quantitate peaks. Each analytical run included three different concentrations of glutamine, citrulline, and arginine standards.

Estimation of villous height

The samples of jejunum and ileum embedded in OCT were sectioned onto glass slides using an Ames Cryostat II (Miles Inc.). Sections were cut perpendicular to the mucosal surface at a depth of 6 μm. Two sections were placed on each slide before they were air dried for 30 min and then fixed for 10 min in acetone. After drying for 10 min the sections were stained for 5 sec with Gill's hematoxylin-eosin, washed with water, and mounted using Kaiser's mounting medium. Villous height was estimated by an observer who was not aware of the source of the sections using a light microscope fitted with an eyepiece micrometer at 125× magnification.

Table 2 The effect of starvation for 2 days on indices of jejunal atrophy and ileal atrophy

	Fed ($n = 6$)	Starved ($n = 6$)	P-value
Jejunum			
Intestinal weight (mg/cm)	74.3 ± 7.5	59.6 ± 7.9	0.01
Mucosal weight (mg/cm)	36.8 ± 7.1	23.8 ± 6.0	0.01
Mucosal protein (mg/cm)	2.85 ± 0.88	1.56 ± 0.48	0.01
Mucosal DNA (μg/cm)	273.1 ± 45.5	154.9 ± 45.5	0.01
Jejunal villous height (μm)	930.0 ± 52.0	646.0 ± 60.0	0.01
Ileum			
Intestinal weight (mg/cm)	71.5 ± 4.6	59.8 ± 5.6	0.01
Mucosal weight (mg/cm)	33.5 ± 5.9	23.2 ± 4.2	0.01
Mucosal protein (mg/cm)	2.14 ± 0.73	1.33 ± 0.25	0.01
Mucosal DNA (μg/cm)	230.2 ± 47.4	120.5 ± 15.1	0.01
Ileal villous height (μm)	291.0 ± 18.0	334.0 ± 19.0	0.16

Values are mean ± SD.

Statistical methods

The mean and standard deviation (SD) were used to describe the data. The Mann Whitney U test for unpaired data was used for inter-group comparisons. Statistical significance was defined as the probability of a type I error of less than 5%.

Results

During the 2-day study period, the rats in the starved group lost weight (−32.2 ± 9.3 g) whereas the rats in the fed group gained weight (17.7 ± 6.4 g; $P < 0.01$). The rats in the starved group had a significantly greater plasma concentration of glutamine than did the fed rats (650.5 ± 48.8 vs. 405.7 ± 20.6 μmol/L, $P < 0.01$). In contrast, there was no significant effect of starvation on plasma concentrations of citrulline (25.2 ± 9.7 vs. 26.1 ± 3.1 μmol/L, $P = 0.52$) or arginine (109.8 ± 11.8 vs. 143.3 ± 26.7 μmol/L, $P = 0.14$).

Indices of jejunal atrophy

The rats in the starved group had a significantly lower jejunal weight, mucosal weight, and villous height than did the rats in the fed group (*Table 2*). The rats in the starved group also had significantly lower mucosal protein and DNA content per centimeter of bowel than did the rats in the fed group. In contrast, starvation did not alter the concentration of protein and DNA in the jejunal mucosa (57.1 ± 6.2 vs. 62.0 ± 5.3 μg protein/mg mucosa, $P = 0.24$; 5.74 ± 1.25 vs. 6.41 ± 1.14 μg DNA/mg mucosa, $P = 0.24$). Starvation had no significant effect on jejunal glutamine and arginine concentrations (data not shown). However, the rats in the starved group had a significantly lower jejunal citrulline concentration than did the rats in the fed group (4.01 ± 1.69 vs. 7.75 ± 1.05 nmol/mg protein, $P < 0.05$).

Indices of ileal atrophy

The ileal data exhibited the same trends as the jejunal data (*Table 3*). Namely, starvation was associated with significant decreases in intestinal weight, mucosal weight, and mucosal protein and DNA content per centimeter of bowel.

Table 3 The effect of starvation for 2 days on *glutaminase* activity in the intestinal mucosa

	Fed ($n = 6$)	Starved ($n = 6$)	P-value
Jejunal mucosa			
Glutaminase activity (μmol/hr/mg protein)	1.97 ± 0.45	1.09 ± 0.34	0.01
Glutaminase activity (μmol/hr/cm bowel)	4.90 ± 1.25	2.03 ± 0.57	0.01
Ileal mucosa			
Glutaminase activity (μmol/hr/mg protein)	1.33 ± 0.32	1.12 ± 0.42	0.48
Glutaminase activity (μmol/hr/cm bowel)	2.07 ± 0.69	1.95 ± 0.91	0.93

Values are mean ± SD.

Starvation had no significant effect on mucosal protein and DNA content per milligram of mucosa (52.9 ± 6.6 vs. 48.9 ± 5.3 μg protein/mg mucosa, $P = 0.31$; 5.57 ± 1.83 vs. 5.30 ± 0.67 μg DNA/mg mucosa, $P = 0.24$), ileal villous height, or intestinal glutamine, arginine, and citrulline content (data not shown).

Activity of glutaminase and glutamine synthetase

Starvation reduced the activity of glutaminase (*Table 3*). Specifically, the rats in the starved group had a significantly lower glutaminase specific activity and glutaminase activity per centimeter of jejunum than did the rats in the fed group. Starvation did not significantly decrease glutaminase activity in the ileum. The rats in the starved group also had a significantly lower glutamine synthetase activity per centimeter of jejunum and ileum than did the rats in the fed group (*Table 4*). In contrast, starvation did not have a significant effect on glutamine synthetase specific activity in the intestinal mucosa.

Distribution and level of glutaminase and glutamine synthetase mRNA

Starvation induced a significant increase in the level of glutaminase mRNA in the jejunal and ileal mucosa (*Figure*

Table 4 The effect of starvation for 2 days on *glutamine synthetase* activity in the intestinal mucosa

	Fed ($n = 6$)	Starved ($n = 6$)	P-value
Jejunal mucosa			
Glutamine synthetase activity (nmol/hr/mg protein)	66.8 ± 8.4	91.6 ± 33.1	0.17
Glutamine synthetase activity (nmol/hr/cm bowel)	170.9 ± 21.7	105.8 ± 13.9	0.01
Ileal mucosa			
Glutamine synthetase activity (nmol/hr/mg) protein)	68.3 ± 8.67	79.7 ± 16.9	0.18
Glutamine synthetase activity (nmol/hr/cm bowel)	162.9 ± 50.6	91.0 ± 23.1	0.01

Values are mean ± SD.

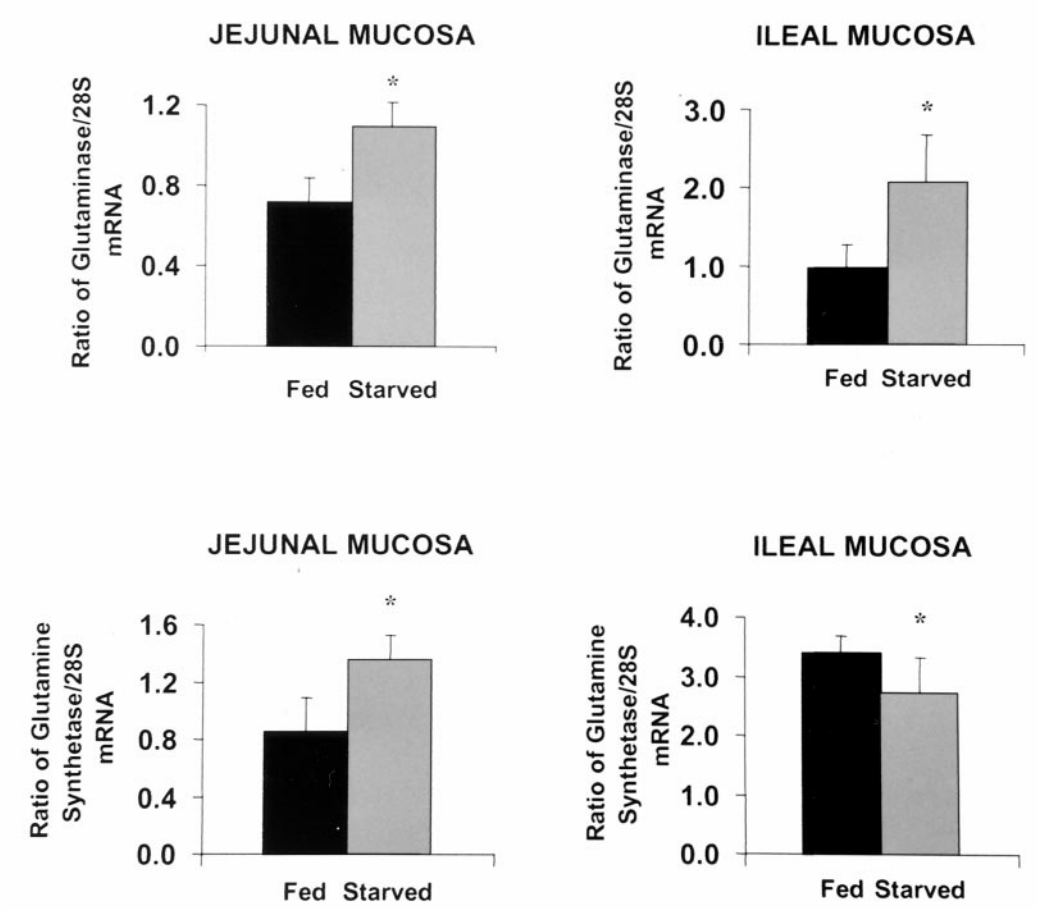

Figure 1 The effect of starvation for 2 days on the level of glutaminase and glutamine synthetase mRNA in the intestinal mucosa. Values are mean ± SD. *$P < 0.05$.

1). Starvation also caused a significant increase in level of glutamine synthetase mRNA in the jejunal mucosa, yet led to a significant decrease in level in the ileal mucosa. Results of the in situ hybridization analysis are shown in *Figure 2*. There was either no staining or very faint staining for glutaminase and glutamine synthetase in the negative control sections (*Figures 2A and 2D*). Glutaminase mRNA was detected in enterocytes throughout the mucosa and it was also detected in monocytes in the villous core of jejunal sections of the starved rats (*Figure 2B*). In contrast, jejunal monocytes in the villous core of rats in the fed group were negative for glutaminase mRNA (*Figure 2C*). Glutamine synthetase mRNA was detected in villous and crypt enterocytes in the jejunum and ileum and there was no effect of starvation on its distribution (*Figures 2E and 2F*).

The results of in situ hybridization analysis of ileal sections were similar to those of jejunal sections (data not shown). Namely, glutaminase mRNA was detected in enterocytes throughout the mucosa and glutamine synthetase was detected in the villous enterocytes. Glutaminase mRNA was not detected in ileal monocytes in the rats of any group. Starvation had no significant effect on distribution of mRNA of either glutaminase or glutamine synthetase in the ileum.

Discussion

The results of our study confirm that starvation has a profound effect on the intestine. Starvation reduces the rate of intestinal cellular proliferation as indicated by decreases in mucosal mass and villous height. Because there is less mucosa per centimeter of bowel, the mucosal weight and mucosal protein and DNA content per centimeter of bowel decrease. In spite of these changes, starvation did not alter the protein and DNA content per milligram of mucosa. In other words, starvation reduces the amount of intestinal mucosa but the composition of the mucosa is unchanged. Hence, it was anticipated that starvation would reduce the activity of glutaminase and glutamine synthetase per centimeter of bowel but would not alter either enzyme activity per milligram of protein or concentration of glutaminase and glutamine synthetase mRNA. Thus, the significant findings in our study were that starvation reduced glutaminase specific activity in the jejunum and that starvation altered the concentration of intestinal glutaminase and glutamine synthetase mRNA. This confirms and extends the findings of Arola et al.,[4] Budohoski et al.,[3] and McFarlane Anderson et al.[17] who found that starvation decreased the activity of glutamine synthetase and glutaminase in the intestine.

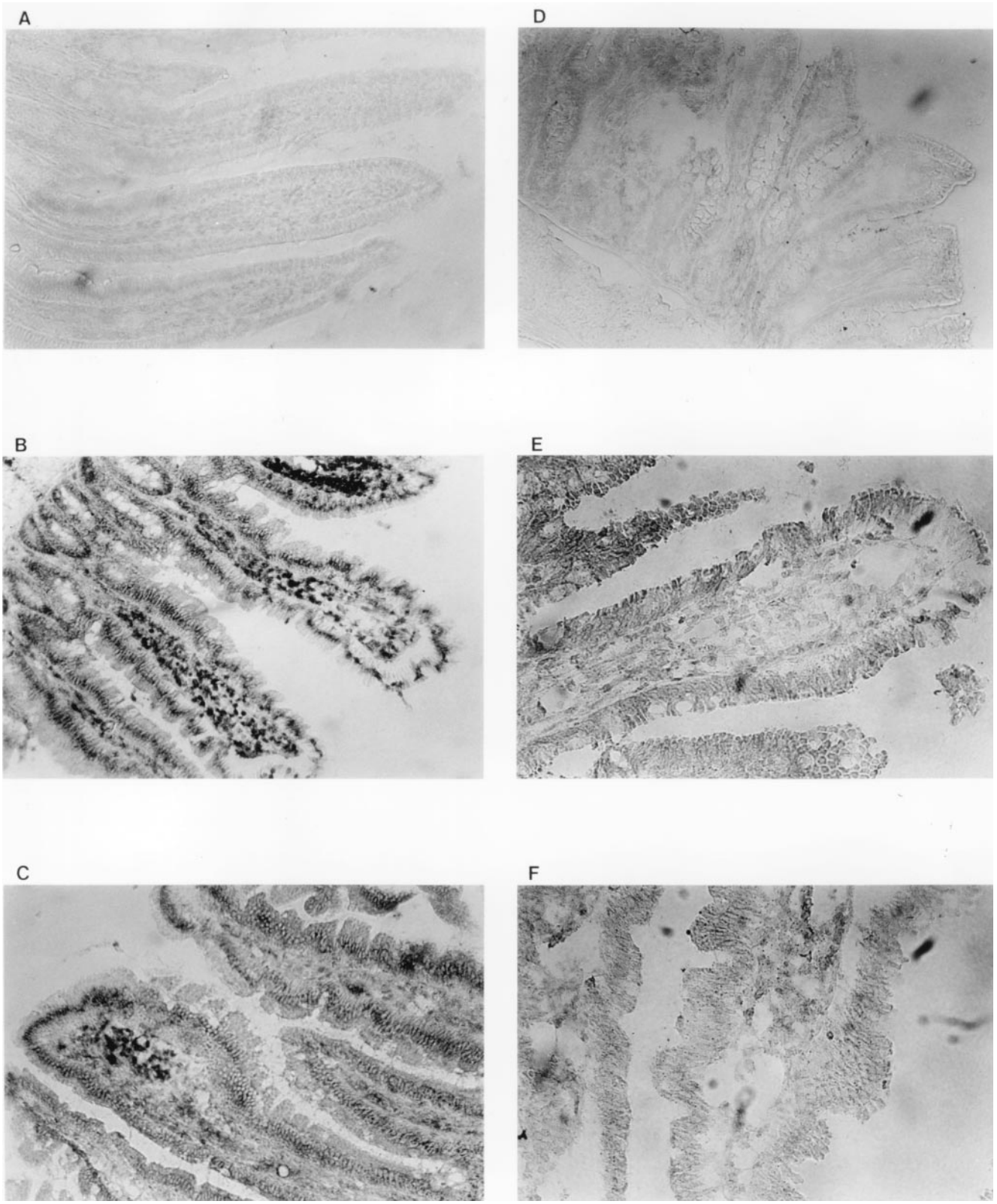

Figure 2 In situ hybridization analysis of the jejunum. (A,D) Negative control sections treated with hybridization buffer containing unlabeled glutaminase or glutamine synthetase probes. (B,C) Distribution of glutaminase mRNA in the jejunum of a rat in the starved and fed groups. (E,F) Distribution of glutamine synthetase mRNA in the jejunum of a rat in the starved and fed groups.

Glutaminase catalyzes the hydrolysis of glutamine to glutamate, ammonia, and water. There are two isoforms of glutaminase and these are derived from different, but related, genes.[18] The "hepatic type" glutaminase is found only in the liver and it is involved in the regulation of the rate of hepatic gluceoneogenesis and urea synthesis. "Kidney-type" glutaminase is active in the intestine, kidney, and many other extra-hepatic tissues. The primary role of glutaminase in the intestine is to regulate enterocyte metabolism. This is because glutamine is the main respiratory fuel of enterocytes[19] and glutaminase catalyzes the rate-limiting step of glutamine degradation.[20] In addition, because ammonia and glutamine can be used for the synthesis of purine and pyrimidine nucleotides it has been suggested that the activity of glutaminase might also be important in the regulation of the rate of synthesis of nucleic acids.[2,21]

Starvation had a different effect on jejunal and ileal glutaminase activity. Starvation decreased glutaminase specific activity and activity per centimeter in the jejunum yet had no significant effect in the ileum. One explanation for this effect of starvation is that the jejunum and ileum have different patterns of nutrient utilization. Ileal enterocytes use butyrate as their preferred respiratory fuel whereas enterocytes use glutamine as their main respiratory fuel.[22,23]

In our study, starvation led to an increase in the level of glutaminase mRNA in the jejunum and ileum. Thus, starvation is able to either increase the transcription rate of glutaminase and/or decrease the rate of degradation of glutaminase mRNA. It seems paradoxical that starvation would increase the level of glutaminase mRNA yet decrease glutaminase activity. A candidate mediator for such an effect is p70 S6 kinase ($p70^{sk6}$). This protein is a serine/threonine kinase that regulates the rate of protein translation. Recently, Iboshi et al.[24] demonstrated that glutamine deprivation of human T-lymphoblast Jurkat cells in vitro inhibits the activity of $p70^{sk6}$.

The effect of starvation on glutamine metabolism and plasma glutamine concentration is controversial. For example, Miller et al.[25] reported that starvation of dogs for 96 hr did not alter the rate of glutamine uptake by the gut and led to an increase in plasma glutamine concentration. In contrast, Cersosimo et al.[26] demonstrated that starvation of dogs for 96 hr led to a doubling of gut glutamine uptake and did not alter plasma glutamine concentration. In spite of these differences, the results from both studies demonstrate that starvation led to an increase in the rate of glutamine uptake by the kidney and switched the liver from net uptake of glutamine to net output of glutamine. In our study fasting rats for 2 days was associated with an increase in plasma glutamine concentration. Thus, our results support the findings of Miller et al.[25] The discrepancies between the experimental results emphasize that the effect of starvation on glutamine metabolism needs further study.

In the fasted rats, intestinal glutaminase activity was decreased in spite of an increase in plasma glutamine concentration. These findings support the observation that an overnight fast halved the rate of glutamine uptake by isolated perfused segments of rat jejunum and led to a 50% decrease in release of ammonia.[1,19] Thus, the effect of fasting on intestinal glutaminase activity seems to be mediated by factors that are independent of plasma glutamine concentration. Further research is needed to unravel the mechanism of effect of starvation on intestinal glutaminase activity.

Glutamine synthetase catalyzes the adenosine triphosphate-dependent conversion of glutamate and ammonia to glutamine. Liver contains the highest specific activity of glutamine synthetase, followed by stomach, brain, kidneys, intestine, skin, and striated muscle.[4] Lie-Venema et al.[27] observed that the main action of intestinal glutamine synthetase might be to provide glutamine for the synthesis of nucleic acids. This is an interesting hypothesis because it implies that exogenous glutamine might not be able to satisfy enterocyte demand. In other words, enterocytes require an exogenous and endogenous source of glutamine. Results from an in vitro study by DeMarco et al.[28] support this idea, as it was found that rat intestinal cells need to be incubated in the presence of 0.46 mmol/L of glutamine to attain half maximal proliferation. Inhibition of glutamine synthetase activity using methionine sulfoximine almost totally inhibited proliferation and this effect of methionine sulfoximine was reversed by the addition of 2.0 mmol/L of glutamine to the culture media. Thus, in this in vitro model, the need of enterocytes for glutamine is met by the combination of endogenous and exogenous sources.

Starvation decreased glutamine synthetase activity per centimeter of intestine yet did not alter glutamine synthetase specific activity. This response is consistent with the postulated role of glutamine synthetase in the intestine. Namely, starvation leads to a decrease in the rate of cellular proliferation[29] and thereby decreases demand for precursors for synthesis of nucleic acids and glutamine synthetase activity. In contrast with the findings of Arola et al.,[4] we did not observe a significant effect of starvation on glutamine synthetase specific activity. Arola et al.[4] demonstrated that starvation for 1 day decreased glutamine synthetase specific activity in the rat intestine. Such contradictory findings may be due to factors such as strain of rat, glutamine synthetase assay method, or methods used to collect the tissue samples.

Starvation increased the level of glutamine synthetase mRNA in the jejunum yet decreased the level in the ileum. One explanation for these results is that jejunum and ileum respond to starvation at different rates. It is possible that the first response to starvation is a decrease in both the rate of enterocyte proliferation and concentration of glutamine synthetase mRNA. During more prolonged starvation, the rate of enterocyte proliferation remains at a low level, whereas the intestine prepares for the resumption of food intake by accumulating glutamine synthetase mRNA. Once the food supply is restored, glutamine synthetase activity will have an important role in the provision of precursors for the synthesis of nucleic acids to support enterocyte proliferation. Support for this hypothesis is provided by the observation that starvation for 2 days alters the rate of enterocyte proliferation in the jejunum, as evidenced by a decrease in jejunal villous height, yet does not alter ileal villous height.

In situ hybridization analysis was used to examine the pattern of distribution of glutaminase and glutamine synthetase mRNA in the intestinal mucosa. Glutaminase mRNA was distributed throughout the intestinal mucosa. These observations confirm the earlier findings of Shenoy et

al.[30] Because there was a substantial difference between the level of glutaminase mRNA in jejunal and ileal enterocytes, this suggests that the level of glutaminase mRNA is greater in jejunal than in ileal enterocytes. However, because there are only minor differences in the intensity of staining for glutaminase in sections derived from rats in the fed and starved groups, it seems that starvation did not alter the distribution of glutaminase mRNA in the intestine. However, it is interesting that starvation was associated with an increase in level of glutaminase mRNA in the monocytes of the jejunal villous core.

In our study, glutamine synthetase mRNA was distributed throughout the mucosa of the jejunum and ileum. In contrast, Roig et al.[31] demonstrated that glutamine synthetase mRNA was mainly localized in the crypt region of the intestine of rats. The type of probe used may be explain this conflict. In our study, a digoxigenin-labeled DNA probe was used, whereas Roig et al.[31] used a digoxigenin-labeled RNA probe. RNA probes are more sensitive than DNA probes because the RNA to RNA bond is stronger than the DNA to RNA bond. Therefore, the sensitivity of the DNA probe may have been insufficient to detect small differences in level of mRNA.

In summary, the results of our study demonstrate that starvation decreases glutaminase specific activity in the jejunum yet increases the level of intestinal glutaminase mRNA. This suggests that starvation leads to a decrease in the rate of intestinal catabolism of glutamine and prepares the intestine for a restoration of food intake by promoting an accumulation of glutaminase mRNA. In addition, our results demonstrate that starvation decreases glutamine synthetase activity per centimeter of intestine. This suggests that starvation decreases the rate of synthesis of glutamine for the assembly of nucleic acids and thereby limits the rate of cellular proliferation.

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