PRELIMINARY REPORT

Functional and Molecular Expression of Intestinal Oligopeptide Transporter (Pept-1) After a Brief Fast

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The intestinal oligopeptide transporter, cloned as Pept-1, has major roles in the assimilation of dietary proteins and absorption of peptidomimetic medications. The initial aim of the present experiment was to investigate whether the functional expression of this transporter is affected by dietary intake. Functional expression was determined as the rate of uptake of glycylglutamine (Gly-Gln) by brush-border membrane vesicles (BBMVs) prepared from the jejunum of fed and fasted rats. Surprisingly, the rate of dipeptide uptake was greatly increased after 1 day of fasting. The subsequent aim of the experiment became the investigation of the mechanism of this alteration in transport, which showed that 1 day of fasting increased (1) the maximal Gly-Gln uptake (V_{max}) by twofold without changing the K_m of Gly-Gln uptake by BBMVs, (2) the amount of intestinal oligopeptide transporter (Pept-1) protein by threefold in the brush-border membrane, and (3) the abundance of Pept-1 mRNA by threefold in the intestinal mucosa. We conclude that 1 day of fasting increases dipeptide transport in rat intestine by increasing the population of Pept-1 in the brush-border membrane. The mechanism appears to be an increase in Pept-1 gene expression.

THE INTESTINAL oligopeptide transporter is responsible for absorption of dipeptides and tripeptides that are the main products of protein digestion in the gut lumen.[1] In addition, the transporter provides an active mechanism for absorption of peptidomimetic agents such as β-lactam antibiotics.[1] These roles have had wide clinical applications, for example, in the use of the oral route for treatment of systemic infection. An important question, which also concerns these applications, is whether the functional expression of the intestinal oligopeptide transporter is affected by dietary intake. The investigation of this question has received a new impetus by the recent cloning of the intestinal oligopeptide transporter designated as Pept-1,[2] because if there is a change in the functional expression of the transporter, it is now possible to determine its molecular basis.

As a first step in the investigation of this question, we have determined the effect of 1 day of fasting on the functional expression of Pept-1 in the rat intestine. Functional expression was studied as the uptake of glycylglutamine (Gly-Gln) by brush-border membrane vesicles (BBMVs), prepared from rat jejunum, in the presence of a proton gradient and membrane potential. Previously, Minami et al[3] validated the use of Gly-Gln for studies of dipeptide transport in the intestinal BBMV. We also used Western and Northern analyses of Pept-1 to investigate the effect of diet on the transporter at the molecular level.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (250 to 300 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Animals were either fed a standard diet or deprived of food for 24 hours before the experiment. All rats had free access to water.

Custom-synthesized [glutamine-3,4-^{3}H]glycylglutamine (49 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Cloned cDNA encoding rat Pept-1 and antibody against rat Pept-1 were provided by Professor Ken-Ichi Inui (Kyoto University, Japan). Filters (type HAWP, 0.45-μm pore size) were purchased from Millipore (Bedford, MA). All other chemicals were purchased from Sigma Chemical (St Louis, MO).

Preparation of BBMVs

Rats were sedated by halothane and then killed by cardiac puncture. After death, the jejunum was immediately removed and the mucosa was scraped. BBMVs were prepared from this freshly obtained mucosal scraping by a magnesium-precipitation technique described previously.[4] Briefly, the tissue scrapings were homogenized in a solution containing 60 mmol/L mannitol, 12 mmol/L Tris hydrochloride, 10 mmol/L EGTA, and 0.1 mmol/L phenylmethylsulfonyl fluoride. Magnesium chloride was added and mixed with the homogenate to a final concentration of 10 mmol/L, and the resulting solution was allowed to stand on ice for 15 minutes (step 1). The suspension was centrifuged at 3,000 × *g* for 15 minutes, and the resulting supernatant was centrifuged again at 27,000 × *g* for 30 minutes (step 2). The pellet from the high-speed centrifugation was resuspended in 35 mL of the above buffer using a Potter-Elvehjem homogenizer. Step 1 and step 2 were repeated on this homogenate, and the resulting pellet was resuspended with the Potter-Elvehjem homogenizer in 10 mL ice-cold preloading buffer (100 mmol/L KCl, 100 mmol/L mannitol, and 20 mmol/L HEPES/Tris at pH 7.4). The final suspension was centrifuged at 27,000 × *g* for 30 minutes. The purified membrane pellet was resuspended in preloading buffer by Potter homogenization for a final protein concentration of 8 to 10 mg/mL. The protein concentration was measured with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). BBMVs were frozen in liquid nitrogen and used the following day.

Transport Measurement

Uptake studies with BBMVs were performed at 37°C using a rapid-filtration technique previously described.[3] BBMVs were preloaded with 100 mmol/L KCl, 100 mmol/L mannitol, and 20 mmol/L HEPES/Tris at pH 7.4 and incubated at room temperature with 50 μmol/L valinomycin for 30 minutes. Gly-Gln uptake was initiated by mixing 20 μL preloaded membrane suspension with 180 μL transport buffer (100 mmol/L choline chloride, 100 mmol/L mannitol, and 20

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mmol/L 2-(*N*-morpholino)ethanesulfonic acid (Mes)/Tris at pH 5) containing 5 to 40 mmol/L unlabeled Gly-Gln and Gly-^{3}H-Gln. Incubation was terminated by injecting 20 μL of the vesicle mixture into 2 mL ice-cold stop solution (same composition as the transport buffer, but without Gly-^{3}H-Gln), followed by filtration. The filters were then washed with 5 mL ice-cold stop solution. Radioactivity associated with the filters was counted in a Beckman scintillation spectrometer (Beckman Instruments, Fullerton, CA). Nonspecific binding of Gly-^{3}H-Gln was determined by adding the transport solution and vesicles directly to the ice-cold stop solution, followed by filtration, washing, and counting. This technique has been used by others for determining nonspecific binding (for example, Reshkin and Ahearn[5]).

Calculations and Statistics

Kinetic constants of Gly-Gln transport were determined by applying a nonlinear regression method to the Michaelis-Menten kinetic equation using GRAFIT software (Sigma), $V = (V_{max} \cdot [S])/(K_m + [S])$, where V is Gly-Gln uptake in nanomoles per milligram protein per 10 seconds, S is the external Gly-Gln concentration in millimoles per liter, V_{max} is maximal Gly-Gln uptake, and K_m is the concentration of S that yields 50% V_{max}. Each rate of Gly-Gln uptake (corrected for nonspecific binding) is reported as the mean ± SEM of three replicates. Significant differences between values were determined by Student's *t* test.

Western Blot Analysis

Identical amounts (100 μg) of BBMVs prepared from the jejunum of fed and fasted rats were suspended in sodium dodecyl sulfate (SDS) buffer (4% wt/vol SDS, 0.5 mol/L Tris hydrochloride (pH 6.8), 20% vol/vol glycerol, and 1% vol/vol captoethanol). Samples were subjected to SDS/10% polyacrylamide gel electrophoresis in the Laemmli system.[6] Resolved proteins were transferred onto nitrocellulose membranes and subjected to immunoblot analyses. The membranes were incubated with polyclonal antibody (1:1,000) raised against Pept-1 protein. The membranes were washed and incubated with the second antibody (peroxidase-conjugated goat antirabbit immunoglobulin G, 1:2,000) as previously described.[7,8] Pept-1 protein in brush-border membrane was detected with the ECL Plus Western blotting system (Amersham Life Science, Arlington Heights, IL). The intensity of bands was quantified by Image PC (Scion, Frederick, MD).

Northern Blot Analysis

For Northern blot analyses, 5 μg poly(A)$^+$ RNA was isolated (Miniribocep Isolation Kit; Collaborative Research, Bedford, MA) from the jejunum of fed and fasted rats. The isolated RNA samples were size-fractionated by electrophoresis in 1.2% agarose gels containing formaldehyde and transferred onto Nytran membranes (Schleicher & Schuell, Keene, NH) by capillary action. After transfer, mRNA was immobilized by radiation with UV light (UV cross-linker; Stratagene, La Jolla, CA). The membranes were then prehybridized overnight at 42°C in prehybridization solution (50% deionized formamide, 0.25 mmol/L Na_2HPO_4 (pH 7.2), 0.25 mmol/L NaCl, 1 mmol/L EDTA, 100 μg/mL heat-denatured herring sperm DNA, 7% SDS, and 0.1% $Na_4P_2O_7 \cdot 10\ H_2O$). Specific ^{32}P-labeled cDNA probes (Pept-1 or β-actin) were made by the random-primer technique using an oligolabeling kit (Amersham Pharmacia Biotech, Piscataway, NJ) and added to fresh aliquots of prehybridization solution. Hybridization was performed at 42°C for 24 to 72 hours. To remove the unbound probe, membranes were washed twice for 20 minutes in each of the following buffers at 42°C: (1) 2× SSC (1× SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate) and 0.1% SDS, (2) 25 mmol/L Na_2HPO_4 (pH 7.2), 1 mmol/L EDTA, and 0.1% SDS, and (3) 25 mmol/L Na_2HPO_4 (pH 7.2), 1 mmol/L EDTA, and 1% SDS. Hybridization signals were visualized by autoradiography with Biomax MS film (Eastman Kodak, Rochester, NY) for 48 to 72 hours at −70°C. Densitometric analyses of the autoradiographs were performed using Image PC (Scion). Membranes initially hybridized to Pept-1 were subsequently hybridized to β-actin to normalize for differences in mRNA loading between wells.

RESULTS

Effect of Fasting

One day of fasting was well tolerated as evidenced by no significant change in body weight. To investigate whether such a brief fast affects dipeptide transport in the intestine, triplicate measurements of the initial rate of Gly-Gln uptake from two separate BBMV preparations of fed and fasted rats were determined. The results showed a significant ($P < .01$) increase in the rate of uptake (0.29 ± 0.03 *v* 0.55 ± 0.03 nmol/mg protein/10 s). The following studies were performed to investigate the mechanism of this alteration in transport.

Mechanism of Transport Alteration

Fasting may increase Gly-Gln uptake by increasing substrate affinity for the oligopeptide transporter or increasing the population of the transporter in the brush-border membrane of intestinal mucosa. To investigate these possibilities, we determined the effect of 1 day of fasting on the kinetics of Gly-Gln uptake by BBMVs as a function of dipeptide concentration. Eadie-Hofstee plots of the data showed the presence of a single transport system in the intestine of fed and fasted rats. Furthermore, the kinetic analysis of this system showed that fasting caused a twofold increase ($P < .01$) in the V_{max} (19.9 ± 1.6 *v* 41.4 ± 2.7 nmol/mg protein/10 s) but had no significant effect on the K_m (39.5 ± 5.11 *v* 54.71 ± 5.3 mmol/L) (Fig 1).

It is pertinent to note that the K_m of Gly-Gln uptake by rat BBMVs is higher than the value previously reported for human BBMVs.[3] This appears to be due to a species difference, because the same has been found regarding the K_m of Gly-Pro uptake by BBMVs of humans and rats.[9,10]

These results eliminated the possibility of a change in the apparent affinity as a mechanism of stimulation of Gly-Gln transport, since the K_m was not changed. On the other hand, the increase in the V_{max} suggested an increase in the population of the transporter. To investigate this possibility, we determined the amount of Pept-1 protein in the brush-border membrane of intestinal mucosa of fed and fasted rats. Western analyses of Pept-1 showed that the amount was greatly increased after 1 day of fasting (Fig 2A). Densitometric analyses of immunoblots showed that the increase was about threefold (Fig 2B).

To investigate whether the mechanism of increase in the protein mass of Pept-1 was pretranslational, we compared the abundance of Pept-1 mRNA in the intestinal mucosal cells of fed and fasted rats. Northern analyses of Pept-1 mRNA showed a pronounced increase (Fig 3A). The increase was quantified by densitometric analyses that showed a threefold increase in the abundance of Pept-1 mRNA in the intestinal mucosa of fasted rats (Fig 3B).

DISCUSSION

This study is the first investigation of the effect of fasting on the function, protein mass, and gene expression of Pept-1 in the intestinal mucosa. The results show rapid and dramatic increases in all of these parameters. These increases could be

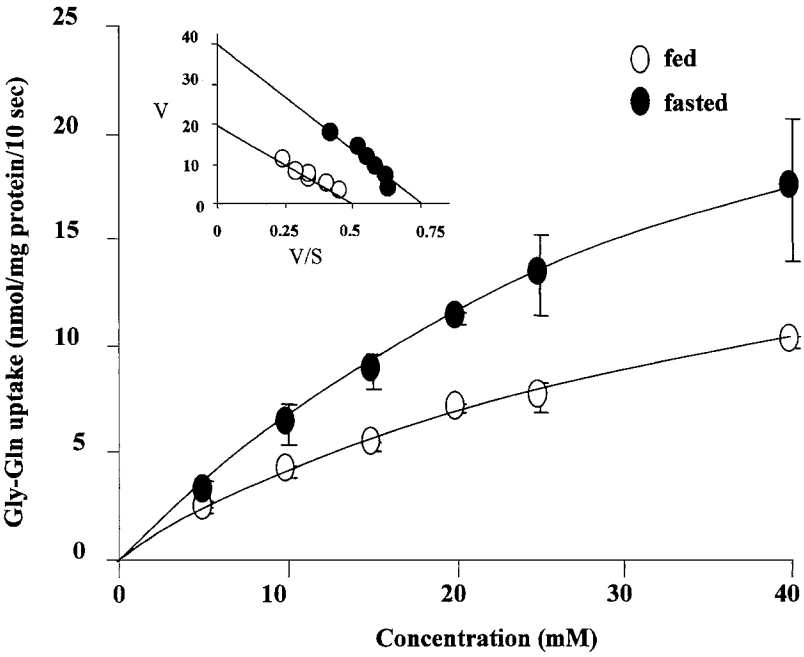

Fig 1. Gly-Gln uptake as a function of substrate concentration. BBMVs prepared from the jejunum of fed and fasted rats were preloaded with 100 mmol/L KCl, 100 mmol/L mannitol, and 20 mmol/L HEPES/Tris at pH 7.4 and preincubated at room temperature for 30 minutes with 50 μmol/L valinomycin. The vesicles were then incubated for 10 seconds with 180 μL transport buffer containing 100 mmol/L choline chloride, 100 mmol/L mannitol, and 20 mmol/L Mes/Tris at pH 5.0 containing Gly-^{3}H-Gln and unlabeled Gly-Gln 5 to 40 mmol/L. Inset: Eadie-Hofstee transformation of the data; V, rate of Gly-Gln uptake (nmol/mg protein/10 s); S, concentration of Gly-Gln in transport buffer (mmol/L).

components of the metabolic adaptation to fasting, which includes measures to minimize loss of body nitrogen. Beginning as early as the first day of fasting, there is increased sloughing of mucosal cells into the gut lumen, as indicated by a decrease in the mucosal weight of the small intestine.[11] This sloughing becomes a major source of loss of body nitrogen. It appears that fasting increases the efficiency of the remaining mucosal cells to absorb dipeptides and tripeptides produced from luminal degradation of the protein content of sloughed cells.

We cannot comment on the uniqueness of the effect of fasting on Pept-1, because there has not been a similar study of the function and gene expression of other intestinal nutrient transporters. However, McManus and Isselbacher[11] have shown that fasting for 1 day significantly reduces glucose and amino acid transport in the rat intestine. Therefore, the effect of 1 day of

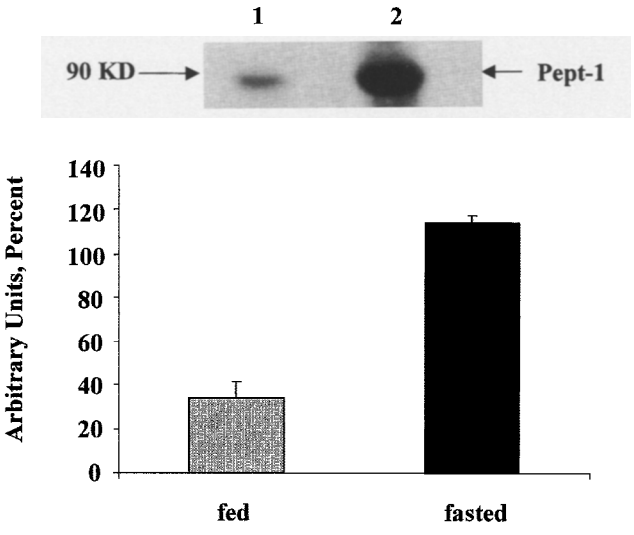

Fig 2. Western blot analysis of Pept-1 protein. Brush-border membranes (100 μg) prepared from the jejunum of fed and fasted rats were subjected to SDS–polyacrylamide gel electrophoresis. For each analysis, brush-border membranes were pooled from 3 rats. (A) Lane 1, fed rats; lane 2, fasted rats. (B) Quantitative densitometric analysis of Western blots for Pept-1 protein. Values are the mean ± SEM of 3 analyses and are expressed as a percentage.

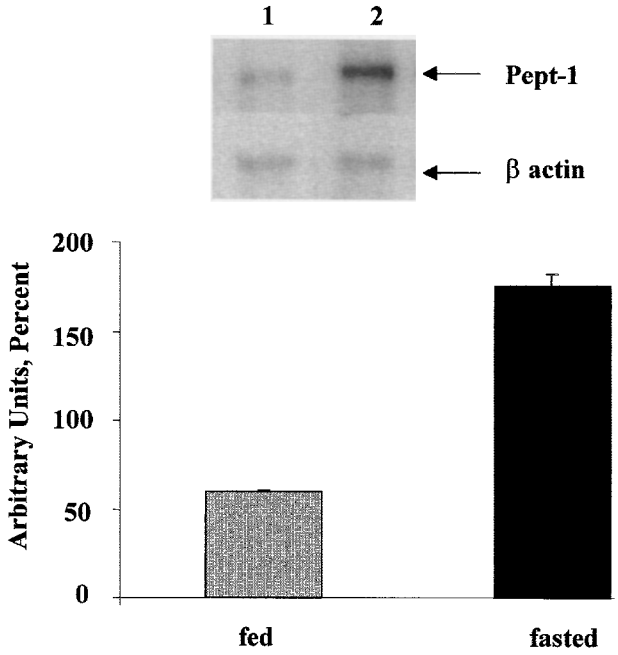

Fig 3. Northern blot analysis of mRNA encoding Pept-1. mRNA was extracted from the jejunum of fed and fasted rats and subjected to Northern blot analysis using ^{32}P-labeled cDNAs encoding Pept-1 and β-actin. (A) Lane 1, mRNA abundance in fed rats; lane 2, mRNA abundance in fasted rats. (B) Densitometric analysis of Northern blots. The level of mRNA for each sample was normalized to the abundance of β-actin mRNA. Values are the mean ± SEM of 3 analyses and are expressed as a percentage.

fasting on dipeptide transport (Fig 1) appears paradoxical to its effect on glucose and amino acid transport.[11] However, this effect does not appear to be unique to fasting, because it has also been observed in other experimental conditions. For example, Tanaka et al[12] recently reported on the regulation of Pept-1 and several other transporters in the rat intestine in response to 5-fluorouracil–induced injury. They found that the injury profoundly decreased the levels of sucrase and Na^+-dependent glucose transporter proteins while having no effect on the amount of Pept-1 protein. Furthermore, in 5-fluorouracil–treated rats, the levels of amino acid, glucose, and phosphate transporter mRNAs were profoundly depressed, whereas the level of Pept-1 mRNA was increased. These observations support a previous report by Matthews and Adibi[13] that the response of oligopeptide transporter to nutritional and pathological conditions is different from the response of amino acid transporters—namely, in adversity one is better preserved than the other.

The results of the present study suggest that the mechanism of the increased population of Pept-1 in the brush-border membrane of mucosal cells is pretranslational. The threefold increase in the protein mass of Pept-1 was accompanied by a threefold increase in the abundance of mRNA encoding Pept-1. These results raise questions about the metabolic signal that increases Pept-1 expression in the intestinal mucosa of fasted rats. Because of the presence of many variables, this question is difficult, if not impossible, to study at the animal level. For this reason, our group and others have been studying the metabolic signals for regulation of Pept-1 in a human intestinal cell line (Caco-2) that allows investigation of an individual metabolic factor. These studies have shown that the addition of insulin[14] and dipeptides[15,16] to the culture medium of these cells stimulates dipeptide transport, while the addition of cyclic adenosine monophosphate (cAMP)[17] inhibits dipeptide transport. These factors cannot account for the stimulation of dipeptide transport observed in the present study, because fasting does not cause an increase in plasma insulin and there was no inhibition of dipeptide transport to implicate cAMP. Therefore, other metabolic factors besides those already identified must be involved in the regulation of dipeptide transport. These, as well as the mechanism of increased gene expression of Pept-1 in fasting, are currently under investigation in our laboratory.

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