Regulation of oligopeptide transporter (Pept-1) in experimental diabetes

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Received 16 October 2001; accepted in final form 21 February 2002

Gangopadhyay, Archana, Manikkavasagar Thamotharan, and Siamak A. Adibi. Regulation of oligopeptide transporter (Pept-1) in experimental diabetes. Am J Physiol Gastrointest Liver Physiol 283: G133–G138, 2002; 10.1152/ajpgi.00445.2001.—The knowledge of expression and biology of the intestinal oligopeptide transporter (Pept-1) in a metabolic disorder such as diabetes may have nutritional and pharmacological implications. To study this problem, rats were made diabetic by streptozotocin injection, and Western and Northern blot analyses and nuclear run-on assays were used to determine the protein and gene expressions of Pept-1 and its rate of transcription, respectively. Uncontrolled diabetes for 96 h increased the activity of Pept-1 in the brush-border membrane of intestinal mucosa. Studies of Michaelis-Menten constant, maximal velocity, and protein expression of Pept-1 indicated that an increase in the abundance of this transporter was responsible for the increased activity. Studies of the gene expression showed that uncontrolled diabetes increased the abundance of mRNA encoding Pept-1 without altering its rate of transcription. Lastly, studies of the specificity of the above effect showed that uncontrolled diabetes similarly affected the protein and gene expressions of Pept-1 located in the kidney. In conclusion, the data show that 1) uncontrolled diabetes has a tropic effect on Pept-1 and 2) the effect is systemic, and its molecular mechanism appears to be an increase in the stabilization of mRNA encoding Pept-1.

MATERIALS AND METHODS

Materials. Custom-synthesized glycyl[3H-3,4]glutamine (Gly-3H-Gln; 49 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Cloned cDNA encoding rat Pept-1 (23) was provided by Ken-Ichi Inui (Kyoto University). The rat β-actin was a gift from Dr. Barbara Attardi (University of Pittsburgh). 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).

Animals and their treatment. Adult male rats (250–300 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Rats were housed in individual cages in air-conditioned quarters with a controlled 12:12-h light-dark cycle, and they received powdered Purina laboratory chow and drinking water ad libitum during the entire period of study. Rats were made diabetic by a single intraperitoneal injection of streptozotocin. The rats were made diabetic by an injection of streptozotocin. Streptozotocin-treated rats have been extensively used by us and other investigators (18) for studies in experimental diabetes. Streptozotocin damages β-cells in the pancreas, and, consequently, its administration reduces insulin secretion, resulting in production of a diabetic-like disorder. However, streptozotocin may affect factors other than insulin secretion. For this reason, we allowed a long period of recovery from the extrapancreatic effect of streptozotocin.

The functional expression of Pept-1 was studied by the uptake of glycylglutamine (Gly-Gln) by brush-border membrane vesicles (BBMV) prepared from the small intestine. The protein and mRNA expressions of Pept-1 were investigated by Western and Northern blot analyses. The rate of gene transcription was measured by nuclear run-on assay.

dependence on an inwardly directed proton gradient for its uphill transport (1).

A major issue that has not yet been studied is the expression and biology of Pept-1 in a metabolic disorder. As a first step in the present experiment, we have investigated the functional and molecular expressions of Pept-1 in the small intestine of diabetic rats deprived of insulin for 96 h. The selection of diabetes as a model of metabolic disorder was prompted by its prevalence in humans and its high clinical relevance.

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Dipeptides and tripeptides, grouped as oligopeptides, are the main products of protein digestion in the gut lumen (3). In addition, many drugs used orally for treatment of common conditions, such as infection and hypertension, have dipeptide- and tripeptide-like structures. The intestinal absorption of these oligopeptides is mediated by a transporter characterized as Pept-1 (1). Pept-1, which is the exclusive peptide transporter in the intestinal brush-border membrane, can also be found in other tissues, such as the brush-border membrane of renal tubules. The function of the renal transporter is to mediate absorption of luminal oligopeptides produced as the result of glomerular filtration of plasma oligopeptides, polypeptides, and proteins (2). The unique features of Pept-1 include

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Oligopeptide Transporter

tozotocin (85 mg/kg body wt) in 0.05 M citrate-buffered saline, pH 4.5. Control rats received a citrate-buffered saline injection. Induction of diabetes was assessed by estimation of blood glucose. Diabetic rats (>2+ glucosuria as measured by Chemstrip; Boehringer Mannheim Diagnostics, Indianapolis, IN) received a daily injection of insulin (Humulin U UltraLente; Eli Lilly, Indianapolis, IN), 2–3 U/day for 8–10 days. At the end of this period, the insulin therapy was withdrawn for 96 h, and then the control and diabetic rats were killed. The rats were sedated by halothane and killed by cardiac puncture. Cardiac puncture was also used to obtain blood for determination of glucose concentration by the glucose-oxidase method. The kidney and small intestine were immediately removed and processed for the studies described below. The small intestine included the segment from 3 cm below the pylorus to 1 cm above the cecum.

For 48 h before the rats were killed, food consumed by each rat was measured each day by subtracting the amount remaining from the amount offered. All of the above procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Preparation of BBMVs. BBMVs were prepared by a magnesium-precipitation technique described previously (9, 27). The purified membrane pellet was resuspended in preloading buffer ([in mM] 100 KCl, 100 mannitol, 20 HEPES-Tris, pH 7.4) by Potter homogenization for a final protein concentration of 8–10 mg/ml. Protein concentration was measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). BBMVs were frozen in liquid nitrogen and used the next day.

Transport measurement. Uptake of Gly-Gln by the intestinal BBMVs was measured by rapid filtration technique, as described previously (16). Briefly, BBMVs were preincubated with the preloading buffer containing 50 μM valinomycin at room temperature for 30 min. Valinomycin was used to generate an inwardly directed negative membrane potential. After preincubation, Gly-Gln uptake was initiated by mixing 20 μl preloaded membrane suspension with 180 μl transport buffer ([in mM] 100 choline chloride, 100 mannitol, and 20 MES-Tris at pH 5.0) containing 5–40 mM unlabeled Gly-Gln and Gly-[3H]-Gln. After 10 s, uptake 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-Gln transport buffer). To remove the unbound ligand, BBMVs were washed with 5 ml ice-cold stop solution, followed by filtration, washing, and counting. The technique has been used by others for determining nonspecific binding (21).

Calculations and statistics. Kinetic constants of Gly-Gln transport were determined by applying a nonlinear regression method to the Michaelis-Menten kinetic equation by using GRAFIT (Sigma)

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V = \frac{(V_{max}[S])}{(V_{max} + [S])}
\]

where \(V\) is in nmol·mg protein\(^{-1}\)·s\(^{-1}\), \([S]\) is external Gly-Gln concentration in mM, \(V_{max}\) is maximal \(V\), \(K_m\) is the concentration of S that yielded one-half \(V_{max}\). Data are means ± SE of at least three replicates. Significant differences between values were determined by Student’s \(t\)-test.

Western blot analysis. Western blot analysis of BBMVs prepared from the small intestine and kidney of control and diabetic rats was performed with Pept-1 antibody as a probe. The following technique was used to prepare Pept-1 antibody. Based on the molecular structure of rat Pept-1, a synthetic peptide (Glu-Asn-Pro-Tyr-Ser-Leu-Glu-Pro-Val-Ser-Arg-Thr-Asn-Met) corresponding to the 15 carboxy-terminal amino acids (696–710) was used as the epitope (23). The rabbit Pept-1 antibody was generated by immunization of rabbit with this epitope. The antibody was further purified by using a HiTrap protein A column (Amersham Pharmacia Biotech). The specificity of antibody was confirmed by Western blot analysis with the purified antibody that had been preabsorbed with the epitope (0.5 μg/ml). BBMVs from intestine and kidney were denatured by boiling for 90 s in sodium dodecyl sulfate (SDS) buffer [4% (wt/vol) SDS, 0.125 M Tris-HCl (pH 6.8), 20% (vol/vol) glycerol, 10% β-mercaptoethanol, 0.5% (wt/vol) bromophenol blue]. Samples were subjected to 10% SDS-polyacrylamide gel electrophoresis in a Laemmli system (14) and transferred onto a nitrocellulose membrane. The membranes were blocked in 10% nonfat dry milk dissolved in Tris-buffered saline containing 0.5% Tween 20 (polyoxyethylenesorbitan monolaurate) (TBST) and washed with TBST. Membranes were incubated with purified polyclonal antibody (1:200) raised against Pept-1 protein for 1 h. Membranes were then washed with TBST and incubated with the second antibody [peroxidase-conjugated goat anti-rabbit IgG (1:5,000)], as previously described (7, 19) and then detected with an enhanced chemiluminescence Western blotting system (Amersham Life Science, Arlington Heights, IL). The intensity of bands was quantified by Image PC (Scion, Frederick, MD).

Northern blot analysis. Total cellular RNA from freeze-clamped small intestine and kidney of control and diabetic rats was extracted by RNAzol method (Tel-Test, Friendswood, TX). The isolated RNA samples (5 μg of intestine and 20 μg of kidney) were size fractionated by electrophoresis in 1.2% agarose gels containing formaldehyde and transferred onto Nytran membranes (Schleider and Schuell, Dassel, Germany) by capillary action. After transfer, RNA was immobilized by radiation with ultraviolet light (ultraviolet cross-linker; Stratagene, LaJolla, CA). The membranes were then prehybridized in Express Hyb (Clontech, Palo Alto, CA) having heat-denatured herring sperm DNA for 2 h at 65°C. Specific 32P-labeled cDNA probes (Pept-1 or β-actin) were made by random-primer technique using an oligolabeling kit (Amersham Pharmacia BioTech, Piscataway, NJ) and added to fresh preheated aliquots of Express Hyb. Hybridization was performed overnight at 65°C. To remove the unbound probe, membranes were washed at 50°C, as described previously (27). Hybridization signals were visualized by autoradiography with Biomax MS film (Eastman Kodak, Rochester, NY) for 5–17 h at −70°C. Densitometric analyses of the autoradiographs were performed as described previously (27).

Isolation of nuclei. The method used for isolation of nuclei from the small intestine and kidney of control and diabetic rats was based on previously published methods (6, 10, 24, 29). The mucosa from freshly obtained intestine was scraped and washed twice with 0.15 M NaCl and 5 mM dithiothreitol (DTT). In the case of the kidney, the tissue was chopped finely. Then the mucosal scrapings and chopped kidney were homogenized separately in ~15 volumes of 0.3 M sucrose buffer A (in mM) 60 KCl, 15 NaCl, 0.15 spermin, 0.5 spermidine, 5 DTT, 0.5 EGTA, 2 EDTA, 15 HEPES (pH 7.5), and 0.1 phenylmethylsulfonyl fluoride). This suspension was homogenized in a Dounce homogenizer by using 10 strokes of loose pestle for gut and 20 strokes of loose pestle for kidney. The homogenate was filtered through four layers of sterile cheese cloth, layered over a 10-ml cushion of 30% sucrose in buffer A, and spun for 10 min at 2,500 revolutions/min in the
The first experiment we performed was to investigate the effect of diabetes on the rate of Gly-Gln uptake by BBMV prepared from the small intestine. At each concentration tested, the rate of uptake was greater by the BBMV of diabetic than control rats (Fig. 1). The concentration used ranged from 5 to 40 mM.

Mechanism of increase in the rate of transport. The mechanisms of increase in dipeptide uptake include either an increase in the substrate affinity for Pept-1 or an increase in the amount of Pept-1 protein. The first possibility was investigated by determination of the $K_m$ of Gly-Gln transport. The results showed no significant difference in $K_m$ of Gly-Gln transport by the BBMV of diabetic and control rats (18.54 ± 6.37 vs. 15.33 ± 5.33 mM). The second possibility was investigated by comparing the $V_{max}$ of Gly-Gln transport by the BBMV of these rats. The results showed that uncontrolled diabetes significantly ($P < 0.01$) increased the $V_{max}$ of Gly-Gln (17.24 ± 2.75 vs. 24.67 ± 3.67 nmol·mg protein$^{-1}·10^3$ s$^{-1}$).

It is pertinent to note that the above $K_m$ values are higher than the $K_m$ value of Gly-Gln in a human intestinal cell line (1.49 mM) we previously reported (28). Several factors, such as species and methodological differences, could contribute to the difference between the $K_m$ values. This also has been observed in previous studies of others. For example, Rajendran et al. (20) and Said et al. (22), who investigated the $K_m$ of glicy prolne in the BBMV of human and rat intestine, reported $K_m$ values of 4.1 and 17.4 mM, respectively.

Because an increase in the $V_{max}$ is only suggestive of an increase in the amount of the transporter, we directly measured the amount of Pept-1 in the brush-border membrane of the intestinal mucosa of the two groups of rats by Western blot analysis (Fig. 2).
results showed that uncontrolled diabetes significantly (P < 0.01) increased the amount of Pept-1 in the diabetic rats. The increase was ~60%.

Mechanism of increase in Pept-1 protein. A possible mechanism for the increased abundance of Pept-1 is an increase in the synthesis of this transporter. This may require an increase in the abundance of mRNA encoding this transporter. To investigate this possibility, we performed a Northern blot analysis of the mucosal cells of the small intestine of diabetic and control rats (Fig. 3). The results showed that, indeed, the uncontrolled diabetes significantly (P < 0.01) increased the abundance of mRNA encoding Pept-1. The increase was ~60%.

Mechanism of increase in Pept-1 mRNA. An increase in mRNA abundance could occur either by increased gene transcription or by increased stability of mRNA. To investigate the first possibility, we performed nuclear run-on assays on the nuclei isolated from the small intestinal mucosa. The results showed that the rates of transcription of the Pept-1 gene were not significantly different in the two groups of rats (Fig. 4). Therefore, an increase in the stability appears to be the mechanism of the increased Pept-1 mRNA abundance in uncontrolled diabetes.

Effect of diabetes on extraintestinal Pept-1. To investigate whether the effect of diabetes on Pept-1 was specific to the intestine or whether it affected other tissues, we performed similar studies on Pept-1 located in the brush-border membrane of renal tubules. The results showed that, like in the intestine, uncontrolled diabetes increased protein (Fig. 2) and mRNA (Fig. 3) expressions of Pept-1 without affecting the rate of its gene transcription (Fig. 4).

DISCUSSION

The results of the present study establish that a metabolic disorder, such as diabetes, has a profound effect on the expression of intestinal Pept-1. In this case, the effect is upregulation of this transporter by increasing its abundance in the brush-border membrane of the intestinal mucosa. Another novel contribution of the present study is that the same is true as far as the expression of Pept-1 in the kidney is concerned. In contrast to intestinal Pept-1, there has been no previous study of the regulation of renal Pept-1.

The simplest explanation for our observation on intestinal Pept-1 could be that uncontrolled diabetes caused hyperplasia of the intestinal mucosa. Although
this was not determined in the present experiment, previous studies have shown that intestinal hyperplasia occurs much later than 96 h of uncontrolled diabetes in rats (15). We chose 96 h mainly to avoid this problem. Furthermore, the above explanation does not take into account the fact that diabetes also increased the expression of renal Pept-1. There is no evidence that uncontrolled diabetes causes hyperplasia in the kidney.

Another possible explanation could be that the uncontrolled diabetes resulted in reduced food intake, causing starvation. In a recent study Thamotharan et al. (27) found that fasting for 24 h greatly increased the abundance of Pept-1 and its gene expression in the brush-border membrane of intestinal mucosa. For this reason the food intake was monitored for 48 h before death. The results showed no decrease in food intake by diabetic rats, eliminating the above possibility.

Reduced insulin secretion in streptozotocin-treated rats triggers a cascade of metabolic alterations. Therefore, a relevant question is whether insulin normally downregulates the Pept-1 expression and, therefore, the decline in its blood concentration may serve as the signal for upregulation of Pept-1 in diabetes. Thamotharan et al. (28) recently studied whether insulin has any role in regulation of Pept-1 located in the apical membrane of a human intestinal cell line (Caco-2). This cell line has been established for in vitro studies of physiology and molecular biology of Pept-1. They found that the addition of insulin in a physiological concentration to the culture medium increased the $V_{\text{max}}$ and protein expression of Pept-1 in this cell line (28). The duration of insulin treatment was 30–60 min. During this period, the abundance of Pept-1 mRNA remained unchanged. But, if the duration of incubation with insulin was increased to 24 h, there was also an increase in the Pept-1 mRNA abundance (Thamotharan and Adibi, unpublished observation). These observations suggest that initially insulin increases the membrane population of the transporter by increasing its translocation from a preformed cytoplasmic pool and then subsequently by increasing its synthesis. In view of these results, it appears less likely that a fall in blood insulin concentration was responsible for the upregulation of Pept-1 in uncontrolled diabetes.

Finally, the metabolic signal for the tropic effect of diabetes on Pept-1 could be an increase in concentrations of oligopeptides and/or amino acids. Oligopeptides and amino acids are the products of both extracellular and intracellular protein degradation (3, 8), and protein degradation is increased in uncontrolled diabetes. Furthermore, the following studies have shown that oligopeptides and/or amino acids are regulators of Pept-1 expression.

Ferraris et al. (12) investigated the uptake of carnosine (a dipeptide) in everted intestinal sleeves of mice whose dietary protein level was varied. They found that, compared with an 18% protein diet, a 72% protein diet increased the uptake of carnosine by 30–70% in the duodenal and jejunal segments. Erickson et al. (11) and Shirago et al. (25) showed a molecular basis for the stimulatory effect of dietary protein on dipeptide transport. They found that increasing the protein content of the diet increases the Pept-1 mRNA abundance in the rat small intestine. Thamotharan et al. (26) hypothesized that dipeptides regulate their own transport. To investigate their hypothesis, they determined peptide transport activity and Pept-1 expression in Caco-2 cells cultured for 24 h in a medium containing a hydrolysis-resistant dipeptide (Glycylsarcosine). They found that the addition of Gly-Sar to the medium increased the $V_{\text{max}}$ of dipeptide transport without altering its $K_{\text{m}}$. The increased transport activity appeared to be due to increased abundance of Pept-1 in the apical membrane of Caco-2 cells brought on by the increased gene expression. In contrast to Gly-Sar, addition of a corresponding mixture of glycine plus sarcosine to the culture medium did not result in any increase in Pept-1 expression. Walker et al. (30) investigated the mechanism of increased abundance of mRNA encoding Pept-1 in Caco-2 cells incubated with a dipeptide. They found that this increase in part was due to an increase in Pept-1 mRNA half-life. Because the increase in half-life did not fully account for the increase in mRNA level, they suggested that an increase in gene transcription must also be involved. Indeed, Shiraga et al. (25), also studying this problem in Caco-2 cells, reported that dipeptides and certain amino acids directly stimulate the transactivation of Pept-1 gene promoter.

The present results suggest that only one of the above two mechanisms, namely increased stabilization of Pept-1 mRNA, was involved in increasing the gene expression, since the transcription of Pept-1, either in the intestine or in the kidney, was not affected by diabetes. It could be that the stabilization of Pept-1 mRNA is more sensitive to metabolic alterations in diabetes than the transcription of this gene. Another possibility is that other factors besides increased protein degradation are involved in regulation of Pept-1 gene expression in diabetes.

There have been very few studies of the effect of uncontrolled diabetes on the molecular biology of nutrient transporters located in the brush-border membrane of intestinal mucosa. In fact, the studies are confined to the Na$^+$-dependent glucose cotransporter (SGLT-1) and the facilitative fructose transporter (GLUT-5). Miyamoto et al. (17) showed that the mRNA expression of SGLT-1 is not affected 2–10 days after the induction of diabetes by streptozotocin injection in rats. On the other hand, if the duration of diabetes was prolonged to 30–60 days, there was a significant increase in the level of this message. Miyamoto et al. did not determine the protein expression of SGLT-1 in diabetes. Burant et al. (4), who investigated both the protein and mRNA expressions of SGLT-1 in the intestine of streptozotocin-induced diabetic rats, found that both expressions were increased.

The data regarding the mRNA expression of GLUT-5 in the intestine of diabetic rats remain controversial. Miyamoto et al. (17) found that diabetes significantly decreases this expression. On the other hand, Kurokawa et al. (13) found no significant change in the expression, and Burant et al. (4) and Castello et al. (5) even found an increase in the expression. However, all investigations of the protein expression of GLUT-5 in
the rat intestine uniformly showed an increase in this expression in diabetes (4, 5, 13).

Therefore, it appears that uncontrolled diabetes results in an increase in a spectrum of nutrient transporters located in the brush-border membrane of intestinal mucosa. However, this increase appears much more rapidly for the peptide than for the sugar transporters. Furthermore, whether the mechanism of increased gene expression is similar for all of the above nutrients cannot be ascertained because the mechanism of increased abundance of mRNA encoding either SGLT-1 or GLUT-5 has not yet been studied. Whatever the mechanism, the up-regulation of these transporters appears to be a useful metabolic adaptation in uncontrolled diabetes. Increased glucose absorption compensates for the loss of glucose in the urine, and increased peptide transport provides substrates for the enhanced gluconeogenesis.

Diabetes is a common metabolic disorder. In light of our recent publication (28) that insulin stimulates the activity and molecular expressions of Pept-1 in an intestinal mucosal cell line, there could be concern about the use of oligopeptides, either as substrates in enteral and parenteral nutrition or as drugs for treatment of conditions such as infection, hypertension, and cancer in diabetic patients. The present data in experimental animals suggest that this concern may not be valid, and in fact diabetic patients may be more efficient in assimilation of oligopeptides. However, this suggestion requires validation in humans.

REFERENCES