Extracellular Glucose Concentration Alters Functional Activity of the Intestinal Oligopeptide Transporter (PepT-1) in Caco-2 Cells

VANESSA M. D'SOUZA,[1] DONNA J. BUCKLEY,[1] ARTHUR R. BUCKLEY,[1,2] GIOVANNI M. PAULETTI[1]

[1]College of Pharmacy, University of Cincinnati Medical Center, 3223 Eden Avenue, Cincinnati, OH 45267

[2]Department of Molecular and Cellular Physiology, University of Cincinnati Medical Center, Cincinnati, OH 45267

Received 21 June 2002; revised 16 September 2002; accepted 28 September 2002

ABSTRACT: The objective of this study was to determine the effect of different cell culture media glucose concentrations on the functional activity of PepT-1 in Caco-2 cells. Uptake kinetics of Gly-Sar into Caco-2 cells that were maintained in iso-osmotic media containing 25 or 5.5 mM glucose were determined in the presence and absence of amino acid-selective chemical modifiers and dithiothreitol. Inhibition of Gly-Sar uptake into Caco-2 cells was measured in the presence of dipeptides and xenobiotics exhibiting various binding affinities for the PepT-1. The effect of extracellular glucose on PepT-1 gene expression was assessed using comparative RT-PCR. Long-term exposure of Caco-2 cells to 25 mM glucose reduced maximum transport capacity for Gly-Sar uptake without altering PepT-1 gene expression. In contrast, binding affinity of Gly-Sar and other dipeptides or xenobiotics was not significantly changed. Chemical modification of Lys and Tyr residues decreased V_{max}, while Cys modification increased the maximum transport capacity of the carrier. Preincubation of Caco-2 cells with dithiothreitol restored PepT-1 activity in cells maintained at 25 mM glucose. In conclusion, cell culture media containing 25 mM glucose decreases maximum transport capacity of PepT-1 in Caco-2 cells without affecting substrate recognition, at least in part, mediated via an oxidative pathway.
 J Pharm Sci 92:594–603, 2003

Keywords: Caco-2 cell; H+/oligopeptide cotransporter; glucose; transport kinetics; oxidation

INTRODUCTION

Various transporters that physiologically enhance transfer of polar nutrients across the intestinal mucosa also facilitate gastrointestinal absorption of drug molecules. Examples include transporters for amino acids, dipeptides, monosaccharides, monocarboxylic acids, organic cations, phosphates, nucleosides, and water-soluble vitamins.[1] As a consequence, novel delivery strategies have been designed that selectively target these intestinal transport proteins with the objective to improve oral bioavailability of drugs.[2,3] The intestinal oligopeptide transporter PepT-1, in particular, has received widespread attention as a promising carrier in modern drug delivery since its recent cloning.[4] PepT-1 plays a pivotal role in assimilation of dietary proteins as well as the absorption of xenobiotics, including β-lactam antibiotics, ACE-inhibitors, and antiviral drugs.[5,6] Moreover, several investigators have demonstrated the utility of the PepT-1 as a platform for improving oral bioavailability of drugs such as zidovudine, acyclovir, ganciclovir, pamidronate, and alendronate through dipeptide prodrug derivatization.[7–9]

Correspondence to: Giovanni M. Pauletti (Telephone: 513-558-4552; Fax: 513-558-0978; E-mail: gm.pauletti@uc.edu)

Journal of Pharmaceutical Sciences, Vol. 92, 594–603 (2003)

Over the past two decades, the Caco-2 cell line has emerged as a powerful *in vitro* model to assess intestinal permeation properties of new chemical entities.[10] In culture, Caco-2 cells differentiate similar to normal enterocytes and form monolayers with physical and biochemical features similar to the intestinal mucosa *in vivo*. Consequently, the Caco-2 paradigm has become an essential tool in drug development to select viable new drug candidates exhibiting acceptable biopharmaceutical/pharmacokinetic properties.[11] Substantial expression of the PepT-1 in the apical membrane of Caco-2 cells renders this *in vitro* system useful for the assessment of substrate activity for this carrier.[12] However, to establish meaningful *in vitro/in vivo* correlations that will allow prediction of the fraction absorbed of a PepT-1 substrate in humans from permeability coefficients determined in the Caco-2 cell culture model, it is critical to understand the various factors regulating PepT-1 activity *in vivo* and *in vitro*. Experimental parameters used in the Caco-2 model that significantly differ from physiological *in vivo* conditions may influence qualitative and quantitative selection criteria for chemical entities, thus leading to inappropriate identification of lead compounds for future drug development. Earlier research has recognized the importance of an inward-directed proton gradient[13] and the influence of epidermal growth factor and hormones such as insulin and thyroid hormone on PepT-1-mediated transport.[14–16]

The objective of this research was to examine the effect of long-term exposure of Caco-2 cells to high extracellular glucose concentrations on the functional activity of PepT-1. Routinely, the Caco-2 cell line is maintained in the presence of 25 mM non-physiological glucose conditions to promote rapid growth and differentiation.[17,18] However, several reports focusing on aging and diabetes mellitus have implicated high extracellular glucose concentrations with significant alterations in regulatory and functional activity of proteins.[19–23] In this study, we determined the maximum transport capacity (V_{max}) and substrate affinity (K_m) of the metabolically stable dipeptide Gly-Sar for intracellular uptake into Caco-2 cells that were maintained in iso-osmotic culture media containing 25 mM (high) and 5.5 mM (physiological) glucose concentrations. In addition, the effect of these different culture conditions on recognition of a series of dipeptides and xenobiotics for the PepT-1 was determined. Furthermore, chemical modification of amino acid residues susceptible to glucose-induced damage was evaluated. Finally, differences in gene expression of PepT-1 in Caco-2 cells maintained under high and physiological glucose concentrations and the effect of the thiol antioxidant dithiothreitol on intracellular Gly-Sar accumulation were assessed.

EXPERIMENTAL

Materials

The dipeptides Gly-Sar, Leu-Met, Ala-Asp, and Gly-Pro were obtained from Bachem Bioscience Inc. (King of Prussia, PA). Cephalexin and cefadroxil were purchased from ICN Biomedicals Inc. (Aurora, OH) and enalapril maleate, bestatin, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), dithiothreitol (DTT), and Hanks' Balanced Salts were purchased from Sigma (St. Louis, MO). *N*-Acetylimidazole (NAI), phenylglyoxal (PG), and *N*-ethylmaleimide (NEM) were obtained from Fisher Scientific (Pittsburgh, PA). [^{3}H]-Gly-Sar (spec. act. = 9.25 GBq/mmol) was obtained from Moravek Biochemicals (Brea, CA). Dulbecco's modified Eagle medium (50-003-PB, 25 mM glucose, 335 ± 30 mOsm/kg and 50-014-PB, 5.5 mM glucose, 335 ± 30 mOsm/kg), L-Glutamine 200 mM (100×), penicillin (10,000 IU/mL), streptomycin (10,000 μg/mL), and nonessential amino acids 10 mM (100×) in 0.85% saline were purchased from Mediatech (Herndon, VA). Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). Bio-Rad® dye reagent concentrate was obtained from Bio-Rad Laboratories (Hercules, CA). All other chemicals were of high purity or analytical grade and used as received.

Cell Culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 18. The cells were routinely maintained in Dulbecco's modified Eagle medium (DMEM) containing either 25 mM (high) or 5.5 mM (physiological) glucose and supplemented with 1% L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 1% nonessential amino acids and 10% heat-inactivated fetal bovine serum at 37°C in a controlled atmosphere of 5% CO_2 and 90% relative humidity. Experiments were performed using Caco-2 cells between passages 57 and 76 that were adapted for at least five passages to the

different glucose concentrations. For Gly-Sar uptake, inhibition, and protein modification studies, 2.2×10^5 cells/well were seeded in plastic 12-well cluster plates (Costar Corporation, Cambridge, MA).

Cellular Uptake Kinetics

Cellular uptake of Gly-Sar into Caco-2 cells that were cultured under high and physiological glucose conditions was determined in triplicate at 37°C. Initially, cell monolayers were preincubated at 37°C with uptake buffer, pH 6.0 containing 25 mM MES, 25 mM Tris, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5.5 mM D-(+)- glucose. After 15 min, the preincubation solution was replaced with 500 μL Gly-Sar solution (0.01–10 mM) prepared in uptake buffer, pH 6.0 that contained a trace amount of [^{3}H]-Gly-Sar. Uptake was terminated after 10 min by the addition of ice-cold Hanks' Balanced Salt Solution (HBSS), pH 7.4. Cell monolayers were dissolved in 1 N NaOH and intracellular Gly-Sar amounts were quantified using a LS-6500 scintillation counter (Beckman Instruments, Fullerton, CA). Total protein content was determined using the Bio-Rad® protein assay. Control experiments performed using uptake buffer, pH 6.0 that contained 5.5 mM glucose and 20 mM mannitol did not reveal a significantly different uptake rate of Gly-Sar into Caco-2 cells (data not shown). Nonspecific binding of Gly-Sar to cell monolayers was determined in the presence of 20 mM Gly-Sar and was found to be $\leq$3%. Inhibition of Gly-Sar uptake into Caco-2 cell monolayers maintained under 25 and 5.5 mM glucose was assessed using various natural dipeptides (i.e., Leu-Met, Ala-Asp, and Gly-Pro) and xenobiotics (i.e., bestatin, enalapril maleate, cefadroxil, and cephalexin), which have published inhibitory constants between 0.1–14 mM.[24,25] Briefly, cellular uptake of 10 μM Gly-Sar was measured in triplicate in the presence of 0.005–10 mM dipeptide inhibitors or 0.01–20 mM xenobiotics using the same protocol as described above.

Determination of PepT-1 mRNA Levels

Total RNA was extracted from $\sim 2 \times 10^7$ Caco-2 cells cultured under high and physiological glucose conditions using guanidine isothiocyanate–phenol–chloroform, followed by precipitation with isopropanol. Subsequently, poly(A)$^+$ RNA was isolated using the Oligotex mRNA spin-column protocol (Qiagen Inc., Valencia, CA). Total RNA (6 μg) or mRNA (0.5 μg) was reverse transcribed using the SUPERSCRIPT™ kit (Life Technologies, Rockville, MD). Two microliters of the resultant reaction mix were then subjected to PCR using Taq DNA Polymerase (Life Technologies, Rockville, MD) in the presence of selective primers for either PepT-1 or human β-actin that served as an internal control. The primers for PepT-1, sense 5′-GCA GTC ACC TCA GTA AGCT-3′ (bp 342–360), and antisense 5′-GCT GCT GAT GTT TGC ATA-3′ (bp 1575–1592),[4] and β-actin, sense 5′-TAC GCC AAC ACA GTG CTG TCT GG-3′ corresponding to nucleotide position 890–912 and antisense 5′-TAC TCC ACT TGC TGA TCC ACAT-3′ corresponding to nucleotide position 1095–1073,[26] were synthesized at the DNA Core Facility, University of Cincinnati (Cincinnati, OH). Amplification was performed within the linear range for 30 cycles according to the following protocol: 94°C for 45 s, 55°C for 45 s, 72°C for 90 s, followed by a postdwell at 72°C for 5 min. PCR products were resolved on a 1.4% agarose gel, stained with ethidium bromide and analyzed by densitometry (Gel Expert 3.5, Nucleotech Corp., San Carlos, CA).

Chemical Modification Studies

For chemical modification experiments, confluent Caco-2 monolayers cultured under physiological glucose conditions were incubated at 37°C for 20–40 min with various selective amino acid modifiers according to a previously published protocol.[27] DIDS (2 mM) was used to modify extracellular Lys residues, whereas Arg residues were modified using PG (20 mM). Protein damage at susceptible Tyr and Cys residues was assessed using NAI (20 mM) and NEM (2 mM), respectively. Modifier solutions were prepared in uptake buffer, pH 7.5 (25 mM HEPES, 25 mM Tris, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5.5 mM D-(+)-glucose) using DMSO as a cosolvent ($\leq$1% v/v). Control cells were incubated with vehicle only. Following preincubation with the modifiers, Gly-Sar uptake kinetics (0.01–10 mM) were measured as described under "Cellular Uptake Kinetics." In addition, Caco-2 cells maintained under elevated glucose conditions were treated for 24 h with the thiol antioxidant DTT (0.1 mM) followed by kinetic characterization of the carrier function using Gly-Sar.

Data Analysis

Substrate binding affinity (K_m), maximum transport capacity (V_{max}), diffusion coefficient (K_d), and inhibitor binding affinity (K_i) were determined by nonlinear regression analysis using the following equations (Prism 2.0, Graph Pad Software Inc., San Diego, CA).

$$V = \frac{V_{max} \times [S]}{K_m + [S]} + K_d \times [S] \quad (1)$$

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}} \quad (2)$$

where V is the velocity of carrier-mediated uptake, V_{max} is the maximum transport capacity, $[S]$ is the concentration of Gly-Sar, K_m is the substrate binding affinity or Michaelis-Menten constant, K_d is the diffusion coefficient, K_i is the apparent inhibition constant and IC_{50} is the inhibitor concentration required to inhibit half ligand-specific binding.

Statistical Analysis

Cellular uptake, inhibition, and chemical modification studies were carried out in triplicate. Results are reported as mean ± SD. Significant statistical differences in the kinetic parameters between two groups were evaluated using an unpaired Student's t-test at the significance level $p < 0.05$.

RESULTS

Gly-Sar Uptake

The metabolically stable dipeptide Gly-Sar exhibits significant substrate activity for the intestinal oligopeptide transporter (K_m ~1 mM) and is commonly used as a model substrate to assess functional activity of PepT-1.[5] To determine whether different glucose concentrations in the culture media influence the characteristic proton-dependent transfer of Gly-Sar into Caco-2 cells, preliminary uptake experiments were performed at pH 6.0 and 7.5 using cells that were maintained in the presence of 5.5 or 25 mM glucose. In both culture conditions, the uptake rate of Gly-Sar was significantly greater in the presence of a proton gradient with linear uptake kinetics of this PepT-1 substrate up to 10 min (data not shown). Because extracellular glucose concentration affects cellular growth and differentiation, Gly-Sar uptake into Caco-2 cells was measured daily over a period of 10 days postseeding. The results in Figure 1 show that maximum functional expression of PepT-1 was reached between days 7 and 9, which appears to be independent of the respective culture media glucose concentration. This time frame is consistent with findings from other investigators who have evaluated the functional activity of PepT-1 in Caco-2 cells grown on plastic support.[12,15] Consequently, uptake studies with Gly-Sar were conducted over an extended substrate concentration range using monolayers maintained for 7–9 days in the presence of high and physiological glucose concentrations.

Intracellular uptake of 0.01–10 mM Gly-Sar into Caco-2 cells cultured under high and physiological glucose conditions exhibits a saturable kinetic pattern, apparently mediated by a single carrier system as revealed by the Eadie-Hofstee transformation (Figure 2). The kinetic parameters estimated for the carrier-mediated process and the passive component of Gly-Sar uptake are summarized in Table 1. High glucose concentration decreased the maximal velocity of carrier-mediated Gly-Sar uptake in Caco-2 cells by 54% ($p < 0.05$). In contrast, binding affinity of this substrate for the PepT-1 was not significantly affected, as revealed by the apparent Michaelis-Menten constants. However, high glucose appeared to significantly increase passive diffusion of Gly-Sar across the apical membrane (Table 1). When cell monolayers were grown in 5.5 mM

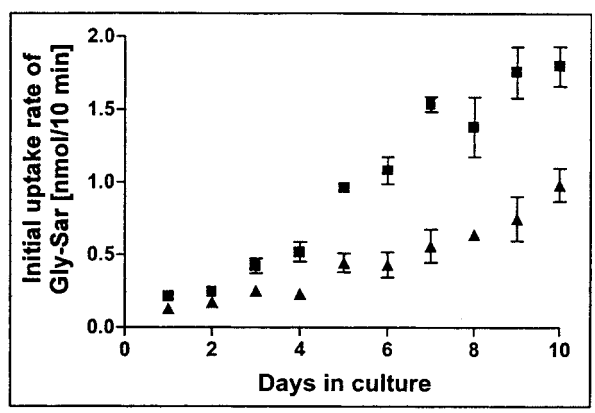

Figure 1. Effect of differentiation and extracellular glucose concentration on PepT-1 activity in Caco-2 cells. Initial uptake rates of Gly-Sar (100 μM) into Caco-2 cells were determined over a period of 10 days using cell monolayers that were maintained under high (triangles) and physiological glucose (squares) concentrations. Values are represented as mean ± SD ($n = 3$).

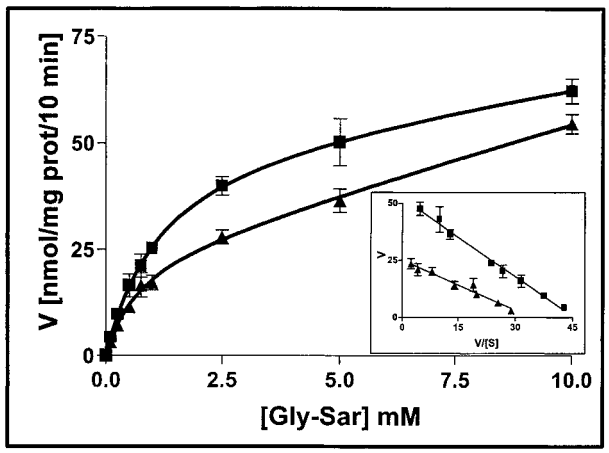

Figure 2. Effect of extracellular glucose concentration on uptake kinetics of Gly-Sar (0.01–10 mM) into Caco-2 cells cultured under high (triangles) and physiological (squares) glucose conditions. Inset: Eadie-Hofstee (V versus V/[S]) transformation of the data.

glucose supplemented with 20 mM mannitol, the kinetic parameters determined for Gly-Sar uptake were not significantly different from those obtained in control cell monolayers cultured in the presence of 5.5 mM glucose alone. These results indicate that long-term exposure of Caco-2 cells to high concentrations of glucose, but not a structurally related compound such as mannitol, reduces maximum transport capacity without altering binding affinity of Gly-Sar for the PepT-1.

To assess the effect of elevated glucose levels on binding of a broad range of substrates for the PepT-1, inhibition of Gly-Sar uptake was determined in the presence of compounds exhibiting affinity constants between 0.1–14 mM for this carrier.[24,25] Apparent inhibition constants (K_i) calculated for the dipeptides and xenobiotics are summarized in Table 2. Compared to the K_m value of Gly-Sar, the dipeptides Leu-Met, Ala-Asp, and Gly-Pro as well as bestatin exhibited greater affinity for the PepT-1. In contrast, enalapril maleate, cefadroxil, and cephalexin showed significantly lower affinity for the carrier system.

Table 2. Inhibition of Gly-Sar Uptake by Dipeptides and Xenobiotics in Caco-2 Cells Cultured Under High and Physiological Glucose

	K_i [mM]	
Inhibitors	25 mM Glucose[a]	5.5 mM Glucose[a]
Leu-Met	0.10 ± 0.01	0.09 ± 0.01
Ala-Asp	0.31 ± 0.08	0.26 ± 0.08
Gly-Pro	0.47 ± 0.05	0.38 ± 0.03
Bestatin	0.59 ± 0.09	0.55 ± 0.05
Enalapril maleate	2.51 ± 0.29	2.05 ± 0.40
Cefadroxil	3.48 ± 0.42	3.58 ± 0.64
Cephalexin	10.34 ± 0.66	11.62 ± 0.55

[a]Mean ± SD ($n = 3$).

Statistical analysis of the K_i values revealed that varying the glucose concentration in the culture media did not significantly affect binding of substrates to PepT-1 irrespective of the affinity constants for the carrier. Furthermore, the rank order for inhibition potency of these molecules was not affected by the glucose concentration. Leu-Met was the most and cephalexin was the least potent inhibitor. These results provide further support for the hypothesis that high extracellular glucose does not interfere with substrate recognition by the PepT-1 in Caco-2 cells.

Determination of PepT-1 mRNA Levels

To assess whether high glucose concentration induces dramatic transcriptional changes of this carrier, mRNA levels of PepT-1 were measured in cells that were maintained in the presence of high and physiological glucose concentrations using comparative RT-PCR (Figure 3). Densitometric analysis of the PCR products showed no significant difference in the mRNA levels of PepT-1 cultured in the presence of 25 or 5.5 mM glucose. This suggests that transcription of PepT-1 is not dramatically altered by increased extracellular glucose. However, differences in protein expression and/or circulation of PepT-1

Table 1. Effect of Extracellular Glucose Concentration on Uptake Kinetics of Gly-Sar into Caco-2 Cell Monolayers

	25 mM Glucose[a]	5.5 mM Glucose[a]	5.5 mM Glucose + 20 mM Mannitol[a]
V_{max} [nmol/mg prot/10 min]	24.77 ± 4.09[b]	53.41 ± 8.83	55.30 ± 4.90
K_m [mM]	0.69 ± 0.23	1.21 ± 0.31	1.12 ± 0.17
K_d [nmol/mg prot/10 min/mM]	3.12 ± 0.42[b]	1.47 ± 0.74	2.81 ± 0.94

[a]Mean ± SD ($n = 3$).
[b]Significantly different from the corresponding physiological glucose (5.5 mM) values ($p < 0.05$).

to the apical membrane from a preformed pool are not ruled out by these results.

Chemical Modification Studies

Hyperglycemia has been shown to selectively alter amino acid residues of proteins, thus, leading to modified functional activity.[19–23] To determine the impact of modified amino acid residues on the functional activity of PepT-1, uptake kinetics of Gly-Sar were measured following preincubation of cell monolayers with various amino acid-selective modifiers. The modifiers were chosen to specifically target amino acids present in the carrier protein that exhibit a high probability to interact with glucose and undergo chemical reactions (i.e., Lys, Arg, Tyr, and Cys). As shown in Table 3, preincubation with the Lys modifier DIDS significantly decreased maximum transport capacity of this carrier by 30%, whereas the Tyr modifier NAI decreased V_{max} by 76%. In contrast, modification of Cys residues using NEM resulted in a 50% increase in transport capacity of the carrier. Treatment of Caco-2 cells with the Arg modifier PG did not affect Gly-Sar uptake.

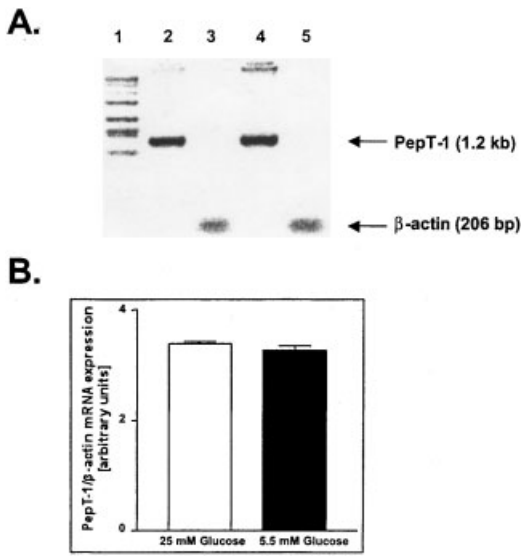

Figure 3. Comparative RT-PCR using primers specific for PepT-1. (A) Shows the effect of glucose on the expression of PepT-1 mRNA in confluent Caco-2 cell monolayers cultured under high (lane 2) and physiologic (lane 4) glucose conditions. Lane 1 represents the size markers (Hi-Lo DNA markers). Lanes 3 and 5 are the respective internal controls using β-actin specific primers for the two groups of cells. (B) Shows densitometric analyses of the PCR products. Values are represented as mean ± SD ($n = 3$).

Interestingly, substrate recognition by the PepT-1 as determined by apparent Michaelis-Menten constants calculated in the presence of these modifiers was not significantly different from control ($K_m = 1.19 \pm 0.24$ mM). These observations support our earlier findings that elevated extracellular glucose does not significantly interfere with substrate recognition by this carrier. However, the findings from this study indicate that Lys, Tyr, and Cys residues are of critical importance for the functional activity of PepT-1 in Caco-2 cells. Because chemical modifications of the oxidation-sensitive residues Tyr and Cys significantly altered functional acivity of PepT-1, the effect of the thiol antioxidant DTT on Gly-Sar uptake kinetics was evaluated using Caco-2 cells maintained under 25 mM glucose. Figure 4 shows that following preincubation with DTT V_{max} increased from 24.77 ± 4.09 to 57.80 ± 7.22 nmol/mg prot/10 min, which is comparable to the maximum transport capacity of this carrier in cells that were maintained under physiological glucose conditions (i.e., 53.41 ± 8.83 nmol/mg prot/10 min). In contrast, binding affinity and apparent diffusion coefficient of Gly-Sar were not significantly affected by treatment with this thiol antioxidant ($K_{mDTT} = 0.79 \pm 0.12$ mM and $K_{dDTT} = 3.34 \pm 0.69$ nmol/mg prot/10 min/mM, respectivley). These results suggest that long-term exposure of Caco-2 cells to high glucose induces oxidative damage in the carrier and/or proteins involved in regulation of functional activity of PepT-1, which are reversible by the presence of a potent thiol antioxidant.

DISCUSSION

The intestinal oligopeptide transporter PepT-1 localized in the apical membrane of enterocytes exhibits broad substrate specificity and has been recognized as a suitable target to improve drug delivery of poorly absorbed drugs.[6–8] Routinely, Caco-2 cells are cultured in the presence of a high glucose-containing culture media.[17,18] Because chronic exposure to high glucose concentration has been suggested to alter regulation and functional activity of proteins and to adversely impact normal cellular processes,[19–23] the focus of this study was to assess whether elevated extracellular glucose concentration affects functional activity of the PepT-1 in Caco-2 cells.

The characteristic proton dependence for carrier-mediated uptake of Gly-Sar provided initial

Table 3. Effect of Chemical Modification of Selected Amino Acid Residues on the Kinetic Parameters of PepT-1 in Caco-2 Cells Cultured Under Physiological Glucose Conditions

	K_m [mM][a]	V_{max} [nmol/mg prot/10 min][a]
Control (vehicle alone)	1.19 ± 0.24	57.80 ± 7.22
Arg-modification	0.70 ± 0.17	46.24 ± 3.78
Lys-modification	1.51 ± 0.28	40.52 ± 4.97^b
Tyr-modification	1.10 ± 0.66	13.90 ± 5.02^b
Cys-modification	1.10 ± 0.21	86.20 ± 9.35^b

[a]Mean $\pm$ SD ($n = 3$).
[b]Significantly different from the corresponding physiological glucose (5.5 mM) values ($p < 0.05$).

evidence that PepT-1 is functionally expressed in Caco-2 cells irrespective of the glucose concentration in the iso-osmotic culture media. Using an extended concentration range of Gly-Sar (0.01–10 mM), kinetic analysis of the carrier-mediated fraction of Gly-Sar uptake showed a significant difference in the maximum transport capacity (V_{max}) as a result of different extracellular glucose concentrations. Because combination of 5.5 mM glucose with 20 mM of the structurally related mannitol did not change the uptake kinetics of this PepT-1 substrate, we conclude that extracellular glucose concentration is uniquely involved in the regulation of intestinal PepT-1 activity. To date, only insulin, thyroid hormone, epidermal growth factor, leptin,[14–16,28] second-messenger molecules such as cAMP[29] and protein kinase C[30] have been implicated as regulators of this carrier protein. It may be possible that extracellular glucose interacts with some of these previously described regulation mechanisms. Our results, however, suggest that the effect of glucose on functional activity of the PepT-1 is most likely mediated by mechanisms other than genetic regulation because PepT-1 mRNA levels were not dramatically affected by extracellular glucose concentrations. Nevertheless, future studies aimed at the involvement of extracellular glucose on regulation of second messengers and PepT-1 expression are warranted.

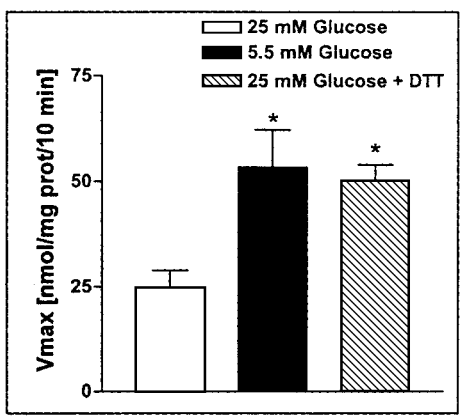

Figure 4. Effect of DTT on maximum transport capacity (V_{max}) of PepT-1 in Caco-2 cells. Uptake kinetics of Gly-Sar (0.01–10 mM) into Caco-2 cells that were maintained under 25 mM glucose were determined after 24 h preincubation with 0.1 mM DTT. Control cells (25 mM and 5.5 mM glucose) were incubated in the absence of the thiol antioxidant. Values are represented as mean $\pm$ SD ($n = 3$). *Significantly different from the corresponding value for cells maintained under increased glucose conditions ($p < 0.05$).

Although the protein configuration at the binding site of PepT-1 still remains subject of intense research, broad substrate specificity implies that this carrier has more than one region involved in substrate recognition. Hence, a structurally diverse array of PepT-1 substrates was used to determine whether binding to the PepT-1 was affected by extracellular glucose concentrations for compounds with varying affinities. The results from these studies demonstrate that non-physiological concentrations of extracellular glucose do not alter interaction between the carrier and its substrates, independent of their respective binding affinities. This implies that the binding site(s) of PepT-1 remain(s) chemically and structurally unaffected by the presence of high extracellular glucose.

Earlier, Cornford and colleagues reported that the maximum velocity of glucose transport across the blood–brain barrier in diabetic mice was significantly lower compared to normoglycemic control animals.[20] The apparent K_m, however, was not different between the two groups. Similarly, sustained hyperglycemia downregulated the GLUT1 transport system in cultured human trophoblasts isolated from term placenta.[21] As compared to normoglycemic conditions, V_{max} decreased by ~50%, whereas K_m remained virtually

unchanged. In cultured retinal pigment epithelial cells, apparent V_{max} of the human taurine transporter (hTT) significantly decreased by 30% without affecting K_m when extracellular glucose concentration increased from 5 to 20 mM.[22] In these cells, the inhibitiory effects of high glucose on hTT appeared to be posttranslationally mediated, because 20 mM glucose decreased hTT mRNA stability without affecting hTT transcriptional rate. In the diabetes literature, it is well documented that high extracellular glucose concentration can lead to spontaneous, nonenzymatic glycation of tissue proteins at basic amino acid residues.[31] Furthermore, reactive oxygen intermediates that result from glucose autoxidation and metabolism have the ability to modify oxidation-susceptible amino acid residues (i.e., Tyr, Cys) and those susceptible to nitration (i.e., Tyr).[19,23] Evaluation of the primary structure of PepT-1 revealed 39 potential targets for such reactions in its predicted five extracellular loops (i.e., 15 Lys, 8 Arg, 11 Tyr, and 5 Cys residues). Selective chemical modification of Lys, Tyr, and Cys significantly altered V_{max} of Gly-Sar uptake suggesting that these residues are crucial for the PepT-1 to facilitate transfer of substrates such as Gly-Sar across the apical membrane of Caco-2 cells. Earlier, site-directed mutagenesis studies revealed that Tyr residues at positions 56, 64, and 167 in PepT-1 play a pivotal role for the functional activity of this carrier.[32,33] These findings are in agreement with our kinetic results. Unfortunately, chemical modification of amino acid residues within PepT-1 or other proteins involved in regulation of functional activity of this carrier solely depends on solvent accessibility and protein orientation. Therefore, it is impossible to draw further conclusions regarding the exact position of a modified amino acid from this study.

The decrease in V_{max} after modification of Lys and Tyr suggests that nonenzymatic glycation of Lys and/or glucose-induced oxidative damage of Tyr residues may be responsible for the observed change in functional activity of the transporter. Experimental support for the hypothesis of glucose-mediated oxidative damage of PepT-1 and/or proteins involved in regulation of functional activity of this carrier was obtained by treatment of cells that were maintained in 25 mM glucose with the thiol antioxidant DTT. The dramatic increase in V_{max} following treatment with DTT to a level that was comparable to the functional activity of PepT-1 in cells cultured in the presence of 5.5 mM glucose implies that oxidation of one of the five Cys residues in PepT-1 and/or Cys residues of proteins involved in regulation of functional activity of this carrier may impair function of this carrier in Caco-2 cells. Earlier, Miyamoto and coworkers reported that treatment of rabbit renal brush-border membrane vesicles (BBMVs), which express the proton-dependent oligopeptide transporter PepT-2, with the thiol-reducing agent dimercaptopropanol stimulated Gly-Sar uptake.[34] The effect of dimercaptopropanol was limited to a change in V_{max} of the carrier system without altering K_m, which is similar to our findings with the PepT-1 in Caco-2 cells. However, treatment of BBMVs with the Cys modifier NEM decreased functional activity of the peptide carrier (PepT-2), whereas maximum transport capacity of PepT-1 in Caco-2 cells increased. This apparent inconsistency may be explained by differences in the molecular structure between the human PepT-1 and the rabbit PepT-2. Extended kinetic studies using various thiol-selective modifiers of different lipophilicity, combined with cysteine-scanning mutagenesis, will provide more conclusive information on the role of selected Cys residues in PepT-1 with respect to functional activity of this carrier in Caco-2 cells.

Nonlinear regression analysis of concentration-dependent Gly-Sar uptake into Caco-2 cells using a modified Michaelis-Menten equation indicated that high glucose increases the apparent diffusion coefficient of Gly-Sar across the apical cell membrane (Table 1). This implies that high glucose concentration modifies physical barrier properties of Caco-2 cells restricting passive diffusion of solutes across the apical membrane. In addition to Gly-Sar, this could potentially affect membrane permeability of protons, which are essential in establishing a transmembrane proton gradient as a driving force for functional activity of PepT-1.[4–6] However, the latter effect appears to be of minor importance because V_{max} of PepT-1 was fully restored after DTT treatment while the apparent diffusion coefficient (K_d) obtained for Gly-Sar was significantly different from control suggesting altered physicochemical properties of the apical membrane under these conditions. Additional studies will be performed using Caco-2 cell monolayers cultured on semipermeable filter support to quantitatively assess the impact of glucose-induced changes in physical barrier properties at the apical membrane on the overall transport of solutes across Caco-2 cell monolayers. These results will be helpful to establish the desired *in vitro*/*in vivo* correlation

to predict the rate and extent of absorption of PepT-1 substrates across the intestinal mucosa *in vivo*.

In conclusion, the results from this study indicate that high concentrations of extracellular glucose reduce maximum transport capacity of PepT-1 in Caco-2 cells without affecting binding of substrates to the carrier, at least in part, mediated via an oxidative pathway.

ACKNOWLEDGMENTS

This research was supported by grants from the University of Cincinnati Research Council (G.M.P.), the National Institutes of Health (DK53452, A.R.B.), and the American Institute for Cancer Research (A.R.B.).

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