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Transport of IRW, an Ovotransferrin-Derived Antihypertensive Peptide, in Human Intestinal Epithelial Caco-2 Cells

Satyanarayana Bejjani and Jianping Wu*

Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5

ABSTRACT: IRW is an egg ovotransferrin-derived ACE inhibitory peptide. The purpose of this study was to evaluate the stability and transcellular transport of IRW in Caco-2 cell monolayers. The stability of IRW was monitored on the apical (AP) surface while its transport was studied from AP to basal (BL) and from BL to AP surfaces. The results revealed that IRW is resistant against intestinal peptidase up to 60 min. Transport of IRW was not affected by addition of wortamanin, a transcytosis inhibitor. However, in the presence of cytochalasin D, a gap junction disruptor, transport of IRW was significantly increased, suggesting a possible passive transport from AP to BL surface. A higher transport of IRW from AP to BL surface than that from BL to AP surface suggests a passive-mediated transport. Moreover, in the presence of glycyl-sarcosine, a substrate for peptide transporter PepT 1, transport of IRW was reduced from AP to BL surface. The above observations showed atypical transport of IRW in Caco-2 cell monolayers. Thus, IRW may possibly be absorbed intact into the site of action for controlling hypertension.

KEYWORDS: *IRW, Caco-2 cells, atypical transport*

INTRODUCTION

Hypertension is a highly prevalent health problem, afflicting ~30% of the adult population worldwide.[1] The major etiological factors for hypertension are age, gender, dietary habits, lifestyle, etc.[2] Use of synthetic drugs has been proven to be effective as the major interventions to control or prevent hypertension but are associated with various adverse effects; thus, there is an increasing interest in the search for safer and less expensive alternatives.[3] Alternatively, many food-based bioactive peptides are being considered to reduce hypertension by inhibiting the activity of angiotensin converting enzyme (ACE). These approaches exploit in vitro digestion conditions to find suitable encrypted sequences from dietary sources to have a positive impact on health.[4,5]

Bioactive peptides from various food sources are reported to exhibit opiate, immunomodulatory, antimicrobial, antioxidant, antithrombotic, and antihypertensive activities.[6−10] Therefore, functional foods containing bioactive peptides represent a novel alternative strategy for the prevention of human diseases. VPP and IPP are two well-studied bioactive peptides from fermented milk. The ACE inhibitory activity of these peptides with blood pressure lowering activity has been well described in vitro and in vivo.[11−13] ACE inhibitory peptides such as FRADHPFL (ovokinin) and RADHFL (ovokinin 2−7) were reported from egg protein ovalbumin.[14−17] Ovotransferrin, which constitutes 13% of egg white, is considered as a multifunctional protein in biotechnological and functional foods applications.[18] Furthermore, ovotransferrin-derived ACE inhibitory peptides were also reported,[19] and three ACE inhibitory peptides, IRW, IQW, and LPK, were identified.[20] IRW has also been shown to exert antioxidant activity in a chemical assay[21] and in cells.[22]

Bioactive peptides, often composed of 2−50 amino acid residues, have attracted great interest for use as therapeutic molecules. These bioactive peptides may function as agonists and antagonists of receptors in disease progression.[23] Nonetheless, bioactive peptides must be absorbed intact to exert their biological function in vivo. Transport of di- and tripeptides as well as many other important drug molecules such as β-lactam antibiotics, orally active ACE inhibitors, and a variety of peptidomimetic drugs are known to be mediated by peptide transporter 1 (PepT 1).[24−28] PepT 1 is present on the apical membrane of the intestine with high affinity toward di- and tripeptides. As a proton-coupled membrane transporter, it belongs to the proton-dependent oligopeptide transporter (POT) family. Human PepT 1 is found throughout the gastrointestinal tract, with the highest level on the apical surface of human epithelial cells for the uptake of di- and tripeptides.[29] ACE inhibitory peptides, YPI, Gly-Pro-Hyp, and VPP, were shown to be transported in the Caco-2 monolayers by active or passive transport.[30−32] In contrast, ACE inhibitory pentapeptides such as VLPVP and HLPLP are passively transported in Caco-2 cells.[33,34] Absorption of di- and tripeptides or oligopeptides involves one or more routes, such as passive transport, active transport, or transcytosis. The absorbed peptides may be either hydrolyzed in cells from peptidase activity or may resist proteolysis and be transported via a basal transporter. Unlike the antihypertensive peptides that have to be absorbed through the intestinal epithelium to reach the target organs, there are peptides that modulate their absorption pathways either by direct effect on the gut lumen (e.g., casien phosphopeptides) or by binding to receptors in the intestine epithelium (e.g., opiod peptides).[4] The absorption and transport of IRW, however, has not been studied. To understand the therapeutic potential of this bioactive peptide, characterization of its stability to intestinal epithelium brush border peptidases is important. Therefore, the objective of this work was to understand the stability of IRW through the

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intestinal epithelium and transcellular transport in Caco-2 cells, a cell line derived from human colorectal carcinoma, which is a well-established model of the small intestinal epithelium.

MATERIALS AND METHODS

Materials. Caco-2 cells (HTB37) were obtained at passage 19 from the American Type Culture Collection (Manassas, VA). Dulbecco's Modified Eagle Medium (DMEM), 0.25% (w/v) trypsin−0.53 mM EDTA, Hanks balanced salt solution (HBSS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), fetal bovine serum, 1% nonessential amino acids, and 1% antibiotics were all procured from Gibco Invitrogen (Burlington, ON, Canada). Triflouroacetic acid (TFA) and acetonitrile (ACN) were purchased from Acros Organics (Morris Plains, NJ). (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT), wortamanin, cytochalasin D, and glycyl-sarcosine (Gly-Sar) were obtained from Sigma (Oakville, ON, Canada). Peptide IRW was synthesized by Genscript Corp (Piscataway, NJ), and its purity >95% was confirmed by high performance liquid chromatography−mass spectrometry (HPLC-MS).

Caco-2 Cell Culture. Cells were grown in DMEM with 25 mM HEPES supplemented with 20% fetal bovine serum, 2 mM glutamine, 1% nonessential amino acids, and 1% antibiotics, at 37 °C in a humidified atmosphere containing 5% CO_2 as described previously.[35] All the cells used in this study were between passages 22 and 35. The cells were subcultured at confluence by trypsin−EDTA treatment before use in the experiments. Cells were seeded onto a six-well Transwell polyester permeable membrane support (0.4 μm pore size, 24 mm diameter, 4.7 cm^2 grown surface, Costar, Corning, NY), at a density of 1×10^5 cells/cm^2. The culture medium was replaced every two days and allowed to differentiate for at least 21 days. Caco-2 monolayers with transepithelial electrical resistance (World Precision Instruments, Sarasota, FL) values higher than 300 Ω/cm^2 were used for the transport studies.

MTT Assay. Cell viability was monitored by MTT colorimetric assay as described previously.[36] Caco-2 cells were grown in 96-well culture plates and incubated with 1−5 mM of IRW at 37 °C for 24 h followed by addition of 10 μL of 5 mg/mL MTT and incubated for 4 h. The cell pellets were dissolved in dimethyl sulfoxide (DMSO), and the absorbance at 595 nm was measured using a plate reader (Molecular Devices, Spectra max, Sunnyvale, CA)

Transport Studies. Caco-2 cells growing in DMEM were placed in HBSS and equilibrated for 30 min at 37 °C prior to transport experiments with minor modifications.[30] Transport of IRW was evaluated by adding peptide at a concentration of 5 mM (dissolved in HBSS buffer) to the AP surface and collecting the BL surface for RP-HPLC analysis. The analysis of the IRW transport from AP surface to BL surface was performed at different time points of 0, 5, 10, 15, 30, and 60 min. In another set of experiment, cells were preincubated with peptide transport PepT 1 substrate Gly-Sar (25 mM in HBSS), or transcytosis inhibitor wortamanin (250 nM in DMSO), or gap junction disruptor cytochalasin D (0.25 μg/mL in DMSO), or DMSO (as control) for 30 min, followed by addition of 5 mM IRW to the AP surface, and transport was studied at 30 min.

RP-HPLC. The AP and BL solutions of IRW from Caco-2 cell monolayers were analyzed on a Waters 600 HPLC system (Waters, Millford, MA) equipped with a 2702 thermoautosampler, a binary gradient pump, and a 2998 photodiode array detector. The column used for the analysis of the AP and BL samples was a C_{18} reversed-phase column (Waters XBridge 250 mm ×3 mm, i.d., 5 μM) protected with a C_{18} guard column (20 mm × 3 mm, i.d., 5 μM) at an injection volume of 25 μL. Mobile phases were solvent A (0.37 mL/L TFA in Milli-Q water) and solvent B (0.27 mL/L TFA in ACN). The peptide was eluted with a gradient of solvent B from 0 to 50% in 15 min, 50 to 100% in 20 min, and 100% B for 5 min at a flow rate of 0.5 mL/min, and the absorbance was monitored at 220 nM as described previously with minor modifications.[37] A linear regression analysis of the peak area vs concentration of a standard solution of the peptide IRW was calculated by using three replicates at seven different concentrations from 1 to 60 μg mL^{-1}. Transport was calculated as the amount of peptide absorbed as the percentage of the total peptide added to the AP or BL chamber.

LC-MS/MS. The IRW sample was subject to LC-MS/MS analysis on a Q-TOF premier mass spectrometer (Waters) coupled with a nanoAcquity UPLC system (Waters). Five microliters of the peptide was loaded onto a nanotrap column (180 μm × 20 mm, Symmetry C_{18} nanoAcquity column) followed by a nanoanalytical column (75 μm × 150 mm, Atlantis d C_{18} nanoAcquity column, Waters). Desalting on the peptide trap column was achieved by flushing the trap column with 1% ACN in water (containing 0.1% formic acid) at a flow rate of 10 μL/min for 2 min. Peptides were separated at a flow rate of 350 μL/min using the following gradient: increasing solvent B (ACN with 0.1% formic acid) from 1% to 5% B in 2 min, to 20% B in 23 min, to 40% B in 15 min, to 65% B in 5 min, and keeping at 65% B for 5 min. The mass spectrometer was operated at a positive mode with a capillary voltage of 3.6 kV and source temperature of 100 °C. Spectra were recorded over the m/z ranges of 400−1400 in MS mode and 50−500 in MS/MS mode. Instrumental control and data analysis were performed using Mass Lynx software (Micromass U.K. Ltd.). Peaks Viewer 4.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) in combination with manual de novo sequencing was used to process the MS/MS data.

Statistical Analysis. Values are expressed as the mean ± SD of three independent experiments using one-way analysis of variance (ANOVA) with the differences at p-values <0.05 considered significant using Graph Pad Prism 5 software (La Jolla, CA).

RESULTS AND DISCUSSION

Cytotoxicity of IRW. The MTT assay showed that IRW did not have any toxicity in Caco-2 cells at the concentrations of 1−5 mM (Table 1), which was comparable to the concentrations of 1−8 mM used for VLPVP.[33] Moreover, the transport experiments were performed within 2 h, which was much shorter than the 24 h for the MTT assay. All the transport experiments were performed at a concentration of 5 mM.

Table 1. Cytotoxcity of IRW in Caco-2 Cells[a]

groups	IRW concentration (mM)	absorbance
1	0	0.696 ± 0.02[a]
2	1	0.696 ± 0.03[a]
3	2	0.685 ± 0.02[b]
4	3	0.678 ± 0.03[c]
5	4	0.679 ± 0.02[c]
6	5	0.680 ± 0.02[c]

[a]All the values are mean ± SD of three determinations. Superscripts with different letters indicate significant differences ($P < 0.05$, $n = 3$).

Stability of IRW in the Apical Surface of Caco-2 Cells. IRW (5 mM) dissolved in HBSS was added to the AP surface of Caco-2 cells to monitor the stability of IRW within 60 min on RP-HPLC. As shown in Figure 1, there was partial hydrolysis of IRW as evident by one additional peak in the RP-HPLC spectrum at an increased time of incubation of 5−60 min. The presence of intestinal peptidases, such as brush border amino peptidases, in the Caco-2 monolayers might cleave N-terminal residues.[26] These results are in agreement with that observed for other tripeptides such as YPI,[30] which were transported into the Caco-2 cells intact after partial hydrolysis, but in contrast with that for VPP[31] and (Gly-Pro-Hyp),[32] which are hydrolyzed to dipeptides and individual amino acids in other intestinal models. Stability of bioactive peptides depends on amino acid composition and positioning within peptides. Peptides containing proline residues at the C-terminal position

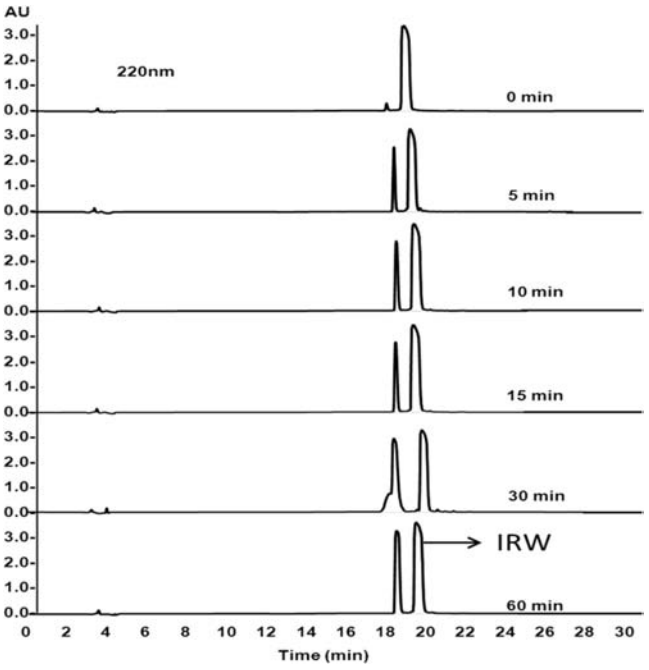

Figure 1. Stability of IRW in the AP surface of Caco-2 cell monolayers. IRW (5 mM) was added to the AP surface, and the stability to intestinal peptidases was monitored by RP-HPLC at time points of 0, 5, 10, 15, 30, and 60 min.

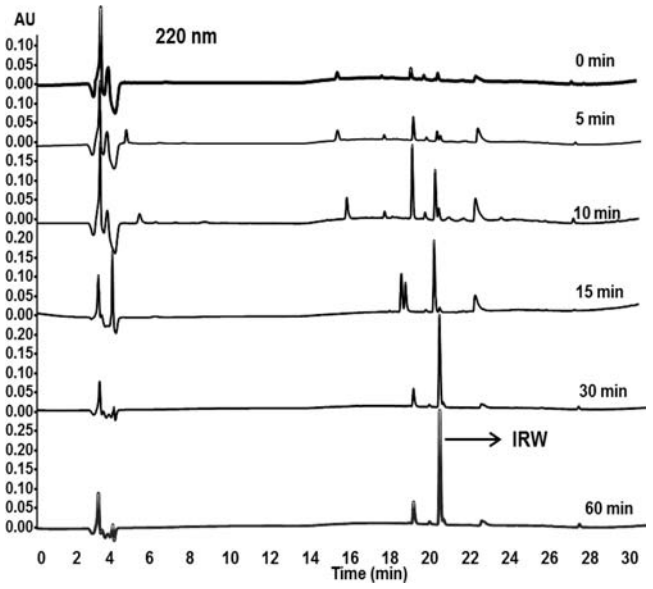

Figure 2. Transcellular transport of IRW from the AP to BL surface was monitored in Caco-2 cell monolayers. IRW (5 mM) was added to the AP surface, and the BL surface analysis was performed at time points of 0, 5, 10, 15, 30, and 60 min by RP-HPLC.

are less susceptible to intestinal peptidases and proteases. Peptides with aromatic residues at the N-terminal position such as YPI,[30] which is produced from YAEERYPIL through in vitro simulated digestion, are stable in Caco-2 cells. This indicates that the hydrolysis is low or milder in peptides containing aromatic amino acids or proline at the N- and C-terminus. The structural requirements for stable peptides are in good alignment with those of potent ACE inhibitory peptides,[38−40] having Pro, Phe, or Trp, Tyr at the C-terminus and branched chain aliphatic amino acids, such as Ile or Val, at the N-terminus. In contrast, peptides such as LAP and LHLPLP are hydrolyzed into individual amino acids.[34] The IRW sequence in the present study is assumed to be a possible potential therapeutic peptide in vivo with Ile at its N-terminus and Trp at its C-terminus, similar to the amino acids in the tripeptides reported above.

Apical and Basolateral Transport. The transport of IRW from the AP to BL surface increased with time (5−60 min) and reached its maximum transport at 60 min as shown in Figure 2. It is evident that there were other peaks in addition to IRW in the BL surface due to partial hydrolysis of IRW on the AP surface. The transport studies for AP to BL surface clearly demonstrated that the absorption of the IRW through the intestinal epithelium of Caco-2 cells is resistant to intestinal peptidase up to 60 min. Though there were additional peaks in the AP to BL surface transported IRW at 5−60 min, the major eluted peak is IRW as evident by RP-HPLC at 60 min.

The BL surface sample at 15 min was further characterized using LC-MS/MS. As shown in Figure 3, the major peak eluted with RP-HPLC from the AP to BL surface sample is IRW. Furthermore, the transport of IRW from AP to BL surface was significantly higher compared to that from BL to AP surface (Figure 4), which indicates that peptide transport is higher from the luminal side to the blood/mesenteric lymph of intestine.

The bioavailability of a peptide through the intestinal monolayers is the first parameter for determining its bioactivity in vivo. Low bioavailability of many bioactive peptides is associated with susceptibility to hydrolysis during intestinal digestion and absorption processes. The bioavailability of several well-studied bioactive peptides, such as YPI[30] and VPP,[31] reported in Caco-2 cells is less than 2%, yet they are effective antihypertensive peptides in vivo. The differences between the in vitro and in vivo bioavailability of antihypertensive peptides is mainly the stability at different sites of absorption and transport to the BL surface. In the present study, the transport of IRW was in the range of less than 1%, which is similar to that for other antihypertensive peptides reported above. The transepithelial transport of intact IRW in the intestinal epithelium with resistance to hydrolysis by peptidases would make it an effective antihypertensive peptide in vivo. We therefore further analyzed the transport mechanisms of IRW with different modulators of peptide transporter 1.

Effect of Various Compounds on IRW Transport. Three pathways of peptide transport are (1) transport by PepT 1, (2) transcytosis, and (3) paracellular transport through intercellular junctions.[26,27,41] The transport of IRW was examined with known modulators of peptides to understand the transport route of IRW. The transport of IRW in the presence of a 5-fold higher concentration of Gly-Sar caused a reduction in the transport of IRW (Figure 5), suggesting a possible PepT 1-mediated transport. It is known that di- and tripeptides are possible substrates for the intestinal peptide transporter PepT 1, which is mainly involved in a degradative pathway as described previously.[30−32] ACE inhibitory peptides such as Gly-Pro-Hyp and YPI are mediated by PepT 1; however, there are peptides, such as VPP, that are not PepT 1 mediated.[30−32] In the present study, however, there was no complete hydrolysis of IRW into individual amino acids and dipeptides after apical uptake and transport in Caco-2 cells. In contrast, the peptides VPP and Gly-Pro-Hyp are reported to be apically taken up in Caco-2 cells and porcine brush border membrane, producing

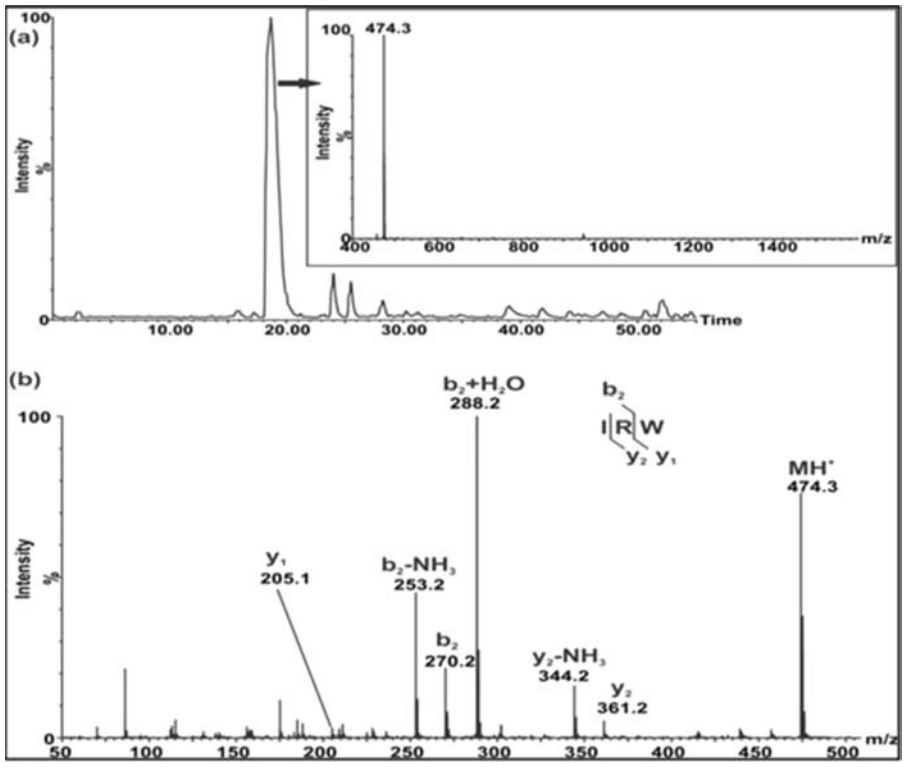

Figure 3. LC-MS/MS characterization of the basolateral solution of IRW at 15 min in Caco-2 cell monolayers. Mass spectrum of IRW (a) and m/z ionization peaks of IRW (b).

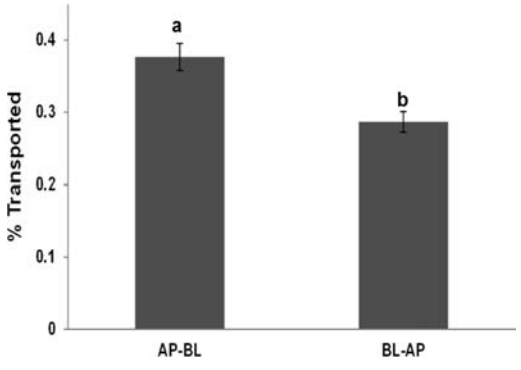

Figure 4. Bidirectional transport of IRW from AP to BL surface and from BL to AP surface in Caco-2 cell monolayers. IRW (5 mM) was added on the AP or BL surface, and the flux of transport was measured by RP-HPLC. Values are mean ± SD of three independent experiments, and bars with different superscript letters differ significantly ($P < 0.05$, $n = 3$).

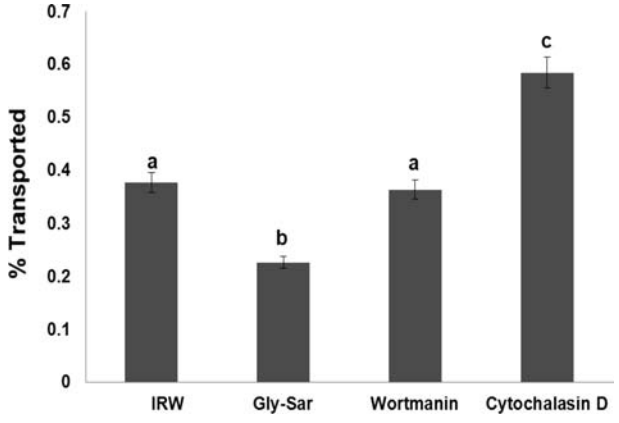

Figure 5. Effects of wortamanin, cytochalasin D, and Gly-Sar on the transport of IRW in Caco-2 cell monolayers. Values are mean ± SD of three independent experiments, and bars with different superscript letters differ significantly ($P < 0.05$, $n = 3$).

free amino acids Val, Pro, and Gly and Pro-Hyp, respectively.[31,32] It should be noted that the transport of small peptides such as IRW in vivo may be higher than that in Caco-2 cells, because it is known that the expression of PepT 1 is low in Caco-2 cells compared to that for intestinal cells of animal origin.[31,42,43]

To understand whether transcytosis is a possible transport mechanism, IRW transport was further studied in the presence of wortamanin, a transcytosis inhibitor. There was no significant effect on the transport of IRW (Figure 5), indicating that transcytosis is not the transport mechanism for IRW. In general, transport of proteins and oligopeptides are mediated by transcytosis; however, the transport of di/tripeptides by transcytosis as the main route of absorption is not clear.

To understand if passive transport is the main mechanism involved in IRW transport, the effect of cytochalasin D was studied. There was a significant increase in IRW transport (Figure 5), which showed that transport of IRW is mediated through the intercellular junctions of the intestinal cells through passive absorption, a nondegradative route of transport that keeps the peptide intact. Various stimulants such as nutrients, interferon-γ,[44] cytokines, and cytochalasin D[45] are known to modulate the tight junctions in the intestinal epithelia, opening up the tight junctions by altering the cytoskeletal structure upon treatment. Furthermore, compounds transported through a passive mechanism in vitro using Caco-2 monolayers would have relatively higher transport in vivo.[46] This indicates that the transport of IRW, which is passively transported in vitro, might

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have higher absorption in vivo. The transport of IRW in the present study is similar to that for VPP, which is apically taken up by PepT 1 and passively transported in Caco-2 monolayers.[31] Thus, IRW, which is resistant to intestinal peptidases on the AP surface of Caco-2 cells, can possibly reach target sites in vivo.

AUTHOR INFORMATION

Corresponding Author

*Tel: 780-492-6885. Fax: 780-492-4265. E-mail: jianping.wu@ualberta.ca.

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Notes

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