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Transport of Insulin in Modified Valia-Chien Chambers and Caco-2 Cell Monolayers

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ABSTRACT The transport characteristics of insulin were investigated using two different absorption models. Using the modified Valia-Chien chambers, permeability coefficients of insulin in the duodenum, jejunum, and ileum were 0.71×10^{-7}, 7.11×10^{-7} and 9.45×10^{-7} cm/s, respectively. In the Caco-2 cell monolayers, the bidirectional transepithelial fluxes of insulin across Caco-2 cell line showed symmetry. Confocal laser scanning microscopy visualized that FD-4 and FITC-insulin were mainly located in the paracellular route. It is evident that the lower intestine might be an advantageous region, and absorption enhancer that helps open tight junctions between cells should be used for oral delivery of insulin.

KEYWORDS Insulin, Transport, Valia-Chien chamber, Protein adsorption, P-glycoprotein, Paracellular pathway

INTRODUCTION

Insulin is a polypeptide drug successfully used in the management of all type I and some type II diabetic patients (Chien, 1996). As compared to subcutaneous injection once or twice each day, currently for clinical application, the oral delivery appears to be the more convenient, socially compatible, and certainly the most physiological method of drug administration, in which insulin could be directly channeled from the intestine to the liver (Ghilzai, 2003). However, the oral route for insulin is impeded by its susceptibility to enzymatic degradation in the gastrointestinal tract and insufficient membrane permeability across the intestinal epithelium (Still, 2002). The oral delivery for insulin cannot be utilized due to its low bioavailability (Alachi & Greenwood, 1994; Bernkop-Schnurch & Walker, 2001).

To effectively develop the oral delivery for insulin, it is important to understand its intestinal permeability including the transport rate, the optimal absorption site, and the transport pathway. The transport behavior of the drug is usually investigated using various in vitro and in situ models, such as perfused animal intestine, excised animal tissues in Ussing chambers, and Caco-2 cell monolayers. Several results have confirmed that these models can properly

predict the in vivo permeability and fraction of the dose absorbed (Lennernäs et al., 1997; Boisset et al., 2000; Zornoza et al., 2004). The cellular morphology and the proteolytic enzyme content of the intestinal tract are so diversified that the selective absorption site of insulin may probably exist in the digestive tract. Much evidence has been reported that insulin can cross the intestinal membrane and shows better absorption from the ileum than the duodenum as well as the jejunum, using everted gut-sac (Schilling & Mitra, 1990), Ussing chambers (Uchiyama et al., 1999), and in situ closed-loop (Morishita et al., 1993). Moreover, human insulin can diffuse through the Caco-2 cell monolayers and its apparent permeability coefficient (P_{app}) is very close to that measured with the everted rat distal jejunum (Greenwood & Al-Achi, 1997).

There are transcellular and paracellular pathways by which a drug moves across the intestinal epithelial barrier, while insulin would be taken up by the latter one due to its high hydrophilicity as is reported by Daugherty & Mrsny (1999). However, immunocytochemical as well as immunochemical evidence has suggested that insulin can be transported transcellularly through the ileum, duodenum, and colon in both normal and diabetic rats (Bendayan et al., 1990, 1994). Using the adenocarcinoma cell line HT-29 derived from human colon, insulin is absorbed by receptor-mediated endocytosis (Sonne, 1985). Consequently, the transport pathway of insulin needs to be further investigated.

The aim of the present study was to investigate the permeability and the transepithelial transport mechanisms of the insulin in modified Valia-Chien chambers and Caco-2 cell monolayers. The relative transport rates of insulin across three different regions of the rat intestine were compared with the modified Valia-Chien chambers that are mainly used in transdermal delivery (Banga et al., 1995; Chen et al., 1995; Kim & Chien, 1996). The Valia-Chien chambers are also utilized in transmucosal delivery such as nasal, rectal and vaginal (Chun & Chien, 1993; Sayani et al., 1994), but not in the oral-enteral delivery. For the Caco-2 cell monolayers, the permeabilities of insulin both apical (AP) to basolateral (BL) and BL to AP directions were measured and the transport pathway of insulin was observed according to the localization of fluorescein isothiocyanate labeled insulin (FITC-insulin).

MATERIALS AND METHODS

Chemicals

Monocomponent insulin crystals (specific activity 28 IU/mg, batch NO. 030305) were purchased from Xuzhou Biochemical Co. (Xuzhou, China). Phenol red was obtained from Shanghai Sanaisi Reagent Ltd Co. (Shanghai, China). Modified Kreb's Ringer phosphate bicarbonate (KRPB) buffer solution (pH 7.4; Schilling & Mitra, 1990) was used in the modified Valia-Chien chambers. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, non-essential amino acids, glutamine, and penicillin-streptomycin were purchased from Gibco (Grand Island, NY). The buffer used in the Caco-2 cell monolayers was a modified Ringer's solution (MRS, pH 7.4) (Saha & Kou, 2002). FITC-insulin and FITC-dextrose (FD-4, MW4400) were obtained from Sigma (St. Louis).

Valia-Chien Method

Male Sprague-Dawley rats weighing 300–350 g in the Animal Centre of Fudan University (China) were used. Animals were given standard food and tap water ad libitum in an air-conditioned room with constant temperature (20 ± 2°C), relative humidity (55 ± 5%), and a standard light/dark cycle (lights on from 8.00 a.m. to 8.00 p.m.). They were received at least 1 week before experiment to enable them to acclimatize. The rats were fasted for 16 hr prior to experiment with free access to water and sacrificed by decapitation. Following a midline incision, various segments of the rat intestine were excised, including duodenum (1 cm distal to pyloric sphincter), jejunum (20 cm distal to pyloric sphincter) and ileum (2 cm proximal to ileocecal junction). The excised tissues were immediately rinsed with ice-cold KRPB solution to remove luminal content. Each segment was opened along the mesenteric line and placed into oxygenated KRPB solution.

The modified Valia-Chien chambers made of glass were used in all experiments. Pieces of intestinal tissue were mounted as flat sheets between the opposing faces of two halves of chambers, in which a surface area of 0.78 cm^2 was exposed. The KRPB solution (5 mL) was added to the serosal side. An equal volume of insulin in modified KRPB buffer solution (1 mg/mL) was added to the mucosal side. Mixing was achieved by bubbling with 95%O_2–5%CO_2 in a steady stream. The diffusion system was maintained at

37°C using a water circulator. At 30, 60, 90, 120, 180, and 240 min, 200 μl samples were withdrawn from the serosal side and an equal volume of blank serosal buffer was added immediately. Sample solution was subjected to centrifugation at 12,000 rpm for 10 min, and the resulting supernatant fraction was used for HPLC analysis. For each test, insulin in the donor solution was also assayed at the beginning and end of the experiment to evaluate potential enzymatic and chemical degradation during the experiment.

The integrity of the intestinal membrane during the test was monitored by measuring the permeability of phenol red. The apparent permeability of phenol red was determined in the three intestinal regions with mucosal solution containing 40 μg/mL phenol red. Phenol red samples were assayed by a UV method.

The apparent permeability coefficient (P_{app}) was calculated from the linear portion of a plot of penetrant accumulated versus time using the following equation:

$$P_{app} = dQ / dT.1 / A.C_0 \quad (1)$$

where Q (mol) is the amount of the drug traversing the tissue in time t (s), A is the exposed area of tissue (cm^2), and C_0 is the initial concentration of drug in the mucosal side (mol/mL).

Caco-2 Cell Monolayers

Cell Culture

The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD). The cells were cultured at 37°C in an atmosphere of 95% air and 5% CO_2 in Dulbecco's Modified Eagle's Medium which was supplemented with 10% heat denatured fetal bovine serum, 1% non-essential amino acids, 2 mM L-glutamine, and 1% penicillin-streptomycin (Krishna et al., 1998; Saha & Kou, 2002). Cells were harvested with trypsin-EDTA and seeded onto polycarbonate Transwell filters (0.4 μm, 4.2 cm^2) at a density of 200,000 cells/cm^2. The culture medium was replaced every 48 hr during the first 5 days and every 24 hr thereafter. The transepithelial electric resistance (TEER) of the monolayer was monitored with a Millicell®-ERS system (Millipore Corp., Boston, Massachusetts, USA). After 18–21 days in culture, only confluent monolayers with TEER values above 200 Ω.cm^2 were utilized for the following experiments.

Selection of Optimum Receiver Medium

To prevent the adsorption of insulin onto the transport apparatus composed of the plastic wells and polycarbonate membranes, several preliminary trials were performed to select the appropriate receiver medium. The modified Ringer's solution (2 mL) containing 0.5% of bovine serum albumin (BSA) were added to the inside and outside of the Transwell inserts, respectively. After 30 min, the solution was removed. The inside and outside of the Transwell inserts were bathed with 2.0 mL of insulin MRS (1 μg/mL) in the absence and presence of BSA with its concentration of 0.1, 0.2, and 0.5%, respectively. The transport apparatus were then agitated at 37°C. A total of 200 μL of fluid was periodically taken from the outside of the Transwell inserts for HPLC assay.

Permeability Studies in Caco-2 Cell

Prior to each experiment, Caco-2 monolayers on Transwell inserts were rinsed three times with MRS and balanced for 30 min with MRS containing 0.5% BSA. For AP–BL transport study, 2.0 mL of insulin MRS (1 mg/mL) was added to the AP (donor) chamber and the BL (receiver) chamber was bathed with 2.0 mL of MRS containing 0.2% BSA. The six-well plates were agitated under 37°C. At 30, 60, 90, 120, 180, and 240 min, 200 μL of sample was withdrawn from the receiver side and an equal volume of receiver medium was added subsequently. The integrity of cell monolayers was assessed by the TEER value at the end of the experiment. The same transport procedure as described for AP to BL was used for BL to AP except that insulin MRS was added to the BL chamber while the AP chamber was bathed with MRS containing 0.2% BSA. The same procedure was repeated using blank membranes without layer of cells. All samples were analyzed by the HPLC method. Permeability values were also calculated according to Eq. (1) except that the exposed area was 4.2 cm^2.

Confocal Laser Scanning Microscopy (CLSM)

An FV-3000 laser scanning microscope (Olympus, Japan) equipped with an argon-ion laser (514 nm) and krypton/argon-ion laser (488 nm) was used. The Caco-2 cell monolayers were rinsed three times with MRS. FITC-insulin solution or FD-4 solution (1 mg/mL) was applied to the apical surface of the cell culture.

After incubation at 37°C for 4 hr, the polycarbonate membranes carefully cut from the inserts were placed on a coverslip, and then mounted in holder system. Images were collected.

Analytical Methods

Insulin was quantified with reversed phase HPLC using a Zorbax 300SB-C18 column (4.6 × 250 mm, 5 micron, Agilent, California, USA). The HPLC system used in this study was made up of an LC 10AT*vp* pump (Shimadzu, Japan), a Rheodyne Model 7725 injection valve equipped with a 20 μL loop (Torrance, CA), a SPD-10A UV-VIS detector (Shimadzu, Japan), and an HS 2000 series of chromatographic workstation (Hangzhou Yingpu Co., Hangzhou, China). The mobile phase was composed of 0.03% acetonitrile and trifluroacetic acid solution (32:68, v/v) at a flow rate of 1.0 ml/min. The UV detector was set at 220 nm. The degradation products and the components of the medium did not interfere with the insulin peak. The amount of phenol red was measured colorimetrically at 555 nm using a U-3000 spectrophotometer (Hitachi, Japan).

RESULTS AND DISCUSSION

Permeability in Various Intestinal Regions of Rats

The everted gut sac technique and the Ussing chamber model have been attempted to evaluate the transport characteristics of peptides and proteins across the intestinal epithelium and simulate this barrier function of the gut (Ungell, 1993). The Valia-Chien chambers are usually used to investigate the transport rate and mechanism of drugs across the skin. Compared to the Ussing chamber model commonly applied in the transmucosal delivery (Boisset et al., 2000; Wadell et al., 2003; Berggren et al., 2004), the exposed area of tissue in the Valia-Chien chamber model is larger and the experimental setup is simpler and more convenient to study insulin transport. Compared with the everted gut sac technique, the Valia-Chien chamber model can effectively prevent peristaltic muscular contractions, which may otherwise alter the shape and internal volume of the sac. In this study, the modified Valia-Chien chambers were applied to exploring the transport of insulin in the rat intestine.

It was important to know how long the intestinal mucosal barrier would remain intact under the conditions of the modified Valia-Chien chambers experimental set up. When the intestinal mucosal cells were destroyed, and aqueous pores in the mucosa would open up, hydrophilic molecules could more easily pass through the enlarged intercellular junctions. Such methods as the constant of transepithelial electrical resistance, accumulation of [^{3}H]-D-glucose, appearance of lactate dehydrogenase and permeability of phenol red were used to determine whether the membranes remained intact throughout the experiments (Bai & Chang, 1996). Table 1 summarizes the permeability experiment of phenol red using the Valia-Chien chambers. After fitting the zero-order equation to the amount of permeability at each sampling time, the regression coefficient was obtained. It was more than 0.99, which indicated that the rate of absorption was constant within 4 hr and the integrity of the intestinal membrane was maintained throughout the experiment with these modified Valia-Chien chambers. The P_{app} of phenol red across the duodenum, jejunum, and ileum were the 4.04×10^{-6}, 4.54×10^{-6}, 5.20×10^{-6} cm/s, respectively. These data were in accordance with those obtained from the Ussing chambers (Bai & Chang, 1996), but lower than those using the everted gut sac technique (Schilling & Mitra, 1990).

Using the Valia-Chien chambers, the in vitro intestinal permeability of insulin was investigated with isolated rat intestinal tissues, including duodenum, jejunum, and ileum (Table 2). It was observed that the amount of insulin in the receiver side increased with time. The P_{app} of insulin across the duodenum, jejunum, and ileum were 0.71×10^{-7}, 7.11×10^{-7} and 9.45×10^{-7} cm/s, respectively. This result indicated the permeability of insulin may vary in different regions of the intestine and the jejunum and ileum appeared to be more permeable to insulin than duodenum. Compared to proximal regions of the small

TABLE 1 **Transport of Phenol Red Across Various Intestinal Membranes Using the Valia-Chien Chambers (n = 4–5)**

Tissue	Apparent permeability coefficient (cm/s, 10^{-6})	r[a]
Duodenum	4.04 ± 0.90	0.999
Jejunum	4.54 ± 0.60	0.997
Ileum	5.20 ± 0.76	0.997

[a]the regression coefficient of the amount of phenol red that transported across various intestinal membranes versus *t*.

TABLE 2 **The Apparent Permeability Coefficient of Insulin Transport Across Various Intestinal Membranes and Residual Amount in the Donor Chamber (n = 4–5)**

Tissue	Apparent permeability coefficient (cm/s, 10^{-7})	Residual amount of insulin in the donor chamber (%)
Duodenum	0.71 ± 0.67	93.63 ± 0.01
Jejunum	7.11 ± 3.80	91.57 ± 0.01
Ileum	9.45 ± 0.97	91.48 ± 0.04

intestine, the distal regions have a smaller surface area and a smaller effective pore radius (Trier & Madara, 1981). These morphological differences seemed to be disadvantageous for absorption of peptide and protein from the distal small intestine. The reason for the regional differences in the transport of insulin was not clearly understood. This might be related to the M cells in the Peyer's patches that were rich in the ileum, which led to a much higher absorption of insulin from the ileum than from the jejunum and duodenum. According to the understanding of permeability of insulin in different intestinal regions, a site-specific delivery technique would be chosen with a view to improving the intestinal absorption of insulin.

Results of the everted gut sac technique indicated that P_{app} for the jejunum (9.08×10^{-7} cm/s) was nearly the same as that for the ileum (7.01×10^{-7} cm/s), but each of them was significantly greater than that for the duodenum (9.39×10^{-8} cm/s; Schilling & Mitra, 1990), which was in accordance with the results in this investigation. However, the permeability of insulin in jejunum and ileum in this study was higher than that using the in vitro Ussing chamber (Asada et al., 1995; Agarwal et al., 2001), which might be due to the differences of test intestinal tissues and experiment protocol.

At the end of the experiment, at least 90–95% of the initial insulin concentration in the mucosal fluid of Valic-Chien chambers was still present at 4 hr, regardless of the duodenum, jejunum, and ileum. This indicated that the insulin degradation due to the proteolytic enzyme did not occur to any extent as to significantly influence the amount of permeability.

Permeability Studies in Caco-2 Cell

Effect of BSA in Receiver

In preliminary experiments, following phenomena were observed: at the beginning of the experiment the insulin concentration in the receiver compartment was higher, which then became the lowest about 1.5 hr later. Subsequently, the amount of insulin increased with transport time. These demonstrated that the adsorption of insulin on the transport apparatus possibly existed.

To prevent adsorption of the transported insulin on the transport apparatus, BSA was added to the receiver compartment. As shown in Fig. 1, the insulin adsorption was significantly reduced when the receiver compartment was balanced with MRS in the presence of 0.5% BSA for 30 min. When the concentration of insulin was 1 μg/mL, it was not adsorbed at 30 min. As the amount of BSA in the receiver compartment increased, the adsorption of insulin decreased. In the presence of 0.2% BSA in the receiver compartment, the insulin was not adsorbed on the transport apparatus and its real transport amount was obtained.

In the Caco-2 cell transport experiments, the transport apparatus consisted of the plastic well and polycarbonate membrane. It was reported that various proteins including bovine serum albumin, lysozyme, ovalbumin, myoglobin, and haemoglobin could be adsorbed on the polymeric microfiltration membranes (polysulfone, polycarbonate, polyvinylidene, cellulose acetate, and mixed esters of cellulose) (Bowen et al., 1995; Güell & Davis, 1996; Kontturi & Vuoristo, 1996) as well as the plastic surface (Sällberg et al., 1995; Clinchy et al., 2003). Insulin was also adsorbed on the polycarbonate membrane and plastic well. Such adsorption would notably influence the measurement of insulin permeability because the P_{app} was very small. During the initial period of transport, concentration of insulin in the receiver compartment was relatively higher

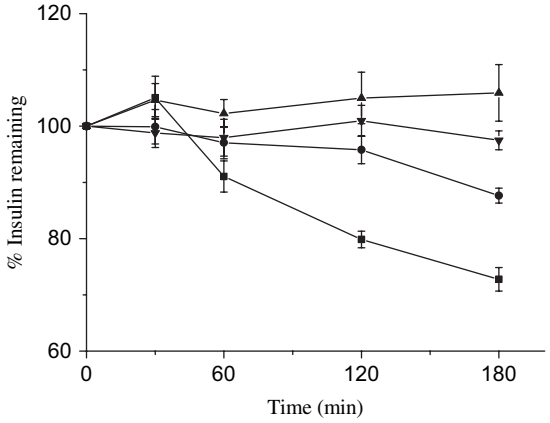


FIGURE 1 **Effect of Various Concentrations of BSA on the Adsorption of Insulin (■) 0; (●) 0.1%; (▲) 0.2%; (▼) 0.5% (n = 3).**

because only a little insulin was absorbed. Along with the achievement of adsorption equilibrium, concentration of insulin decreased to almost zero. Finally, a decrease in adsorption resulted in an increase in the insulin concentration. It was experimentally found that a strongly adsorbing protein could be used to modify the membrane matrix so that adsorption of other proteins was significantly reduced (Kontturi & Vuoristo, 1996). In this study, BSA preferentially adsorbed on the transport apparatus was added to the receiver buffer to prevent adsorption of the insulin.

Permeability in Caco-2 Cell Monolayer

The Caco-2 cell line well-differentiated expresses some characteristics associated with normal intestinal cells, such as the microvillous structure (Brandsch et al., 1994) and the tight junctions at the apical side of the monolayer (Yamada et al., 1992). Furthermore, P-glycoprotein is known to be overexpressed in the apical surface of Caco-2 cells and has been demonstrated to function as an energy-dependent efflux pump for a variety of cytotoxic drugs, other hydrophobic compounds, and some peptide and proteins, such as cyclosporine, valinomycin and a synthetic peptide (Hunter et al., 1993a,b; Sharma et al., 1992).

The P_{app} of insulin was listed in Table 3. The BL–AP/AP–BL permeabilities ratio was close to unity, which indicated that the transport of insulin in the Caco-2 cell monolayers was symmetric between AP–BL and BL–AP directions. Since the P-glycoprotein substrates were commonly hydrophobic, probably no P-glycoprotein was involved in the absorption process of the hydrophilic insulin. However, the transport mechanism may alter with novel delivery agents (Wu & Robinson, 1999). The P_{app} of insulin through the blank membranes was the order of 1.6×10^{-5} cm/s. It was about 80 times higher than those through the Caco-2 cell monolayers, which indicated the blank membranes did not function as a barrier for the transport of insulin.

The apparent permeability values of insulin in Caco-2 cell transport studies were markedly higher than those determined in the duodenum of rats but lower than in the jejunum and ileum with either everted gut sac or modified Valia-Chien chambers. These differences may be explained in terms of the morphological specificity in the thickness of mucous layers, tightness, and number of tight junctions and membrane components of duodenal cells (Delie & Rubas, 1997; Yee, 1997). Compared to those data reported by Greenwood & Al-Achi (1997), the results in this study were relatively low, which might be ascribed to the differences of experiment protocols and cell cultures.

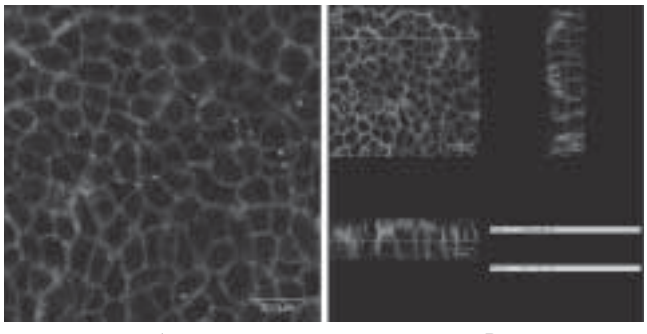
Transport Pathways Across the Caco-2 Cell Monolayers

CLSM was used for the visualization of the FD-4 and FITC-insulin transport pathways. As shown in Fig. 2, the fluorescence appeared only in the intercellular spaces, which indicated a paracellular transport route. This was consistent with the hydrophilic nature of the FD-4 and the data previously reported (Hoogstraate et al., 1994). For the FITC-insulin, the same result was obtained (Fig. 3). The transport pathway of FITC-insulin in the Caco-2 cell monolayers might be paracellular, which was in agreement with the previous study (McRoberts et al., 1990).

Bendayan et al (1990, 1994) reported that insulin could be transported transcellularly through the ileum, duodenum, and colon in normal and diabetic rats. This may be due to the application of sodium cholate perturbing the cell membrane (Meaney & O'Driscoll, 2000). Insulin was absorbed by receptor-mediated

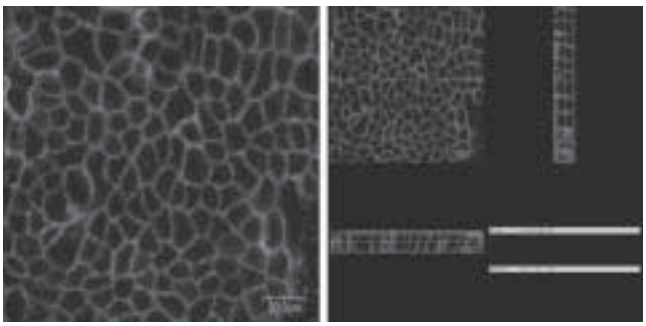
TABLE 3 **The Apparent Permeability Coefficient of Insulin Transport Across the Caco-2 Monolayers and Residual Amount in the Donor Chamber (n = 3)**

	Apparent permeability coefficient (cm/s, 10^{-7})	Residual amount of insulin in the donor chamber (%)
AP→BL	2.39 ± 0.54	100.1 ± 0.01
BL→AP	2.24 ± 0.41	99.38 ± 0.01



A B

FIGURE 2 **CLSM Imaging of FD-4 in the Caco-2 Monolayer Cells. A: X Direction; B: XYZ Direction.**



A B

FIGURE 3 **CLSM Imaging of FITC-Insulin in the Caco-2 Monolayer Cells. A: X Direction; B: XYZ Direction.**

endocytosis in HT-29 cell line (Sonne, 1985). This may be explained by the difference of insulin receptor between in Caco-2 and HT-29 cell surface. The transport pathway of insulin implied that absorption enhancer that helped in inhibiting the enzyme in the cytosol as well as opening the tight junction between cells should be used.

CONCLUSION

The permeability of insulin in the jejunum and ileum was about 10 times than that in the duodenum by the modified Valia-Chien chambers. From the Caco-2 cell monolayers, the transport of insulin was symmetric between AP–BL and BL–AP directions, which suggested that probably no P-glycoprotein was involved in the transport of insulin. Insulin may be transported by paracellular route in the Caco-2 cell monolayers according to the localization of FITC-insulin.

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