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ScienceDirect
International Journal of Pharmaceutics 354 (2008) 143–148
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Confocal microscopic analysis of transport mechanisms of insulin across the cell monolayer

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Received 6 September 2007; received in revised form 3 December 2007; accepted 5 December 2007
Available online 23 December 2007

Abstract

Development of oral insulin formulations would significantly improve the quality of life of patients suffering from diabetes. Complexation hydrogels developed in our laboratory, are one of the most promising classes of materials for use in targeted oral delivery of proteins. Results from confocal microscopy analysis of insulin transport in Caco-2 cells indicated that the primary route of transport was the paracellular pathway and that the transcellular component of the transport was insignificant.

Keywords: Hydrogels; Caco-2 cells; Confocal microscopy; Insulin; Transport

1. Introduction

The transport of therapeutic agents such as proteins across the intestinal gut wall may take place via various pathways (Balimane et al., 2000). The transport can occur primarily through the cell membrane of the enterocytes (transcellular transport) or via the tight junctions between the cells (paracellular transport). The transcellular passive diffusion pathway is mostly limited to drugs that are non-polar, and are lipid soluble, are not charged at the physiological pH of the small intestinal lumen. The octanol–water system is typically used as a reference system for biological partitioning in drug design work. Drug lipophilicity is estimated by determining the drug's octanol–water partition coefficient (Smith et al., 1975; Garcia et al., 2001). Insulin has low lipophilicity with an octanol–water partition coefficient of about 0.0215 (Lee, 1988). Further, the iso-electric point of insulin is around 5 and hence insulin is negatively charged at the neutral pH of the small intestine. Thus, entry into the cell membrane is unfavorable. The primary pathway available for transport of insulin across the epithelium is the aqueous paracellular pathway (Peppas et al., 2000; Peppas, 2006; Peppas et al., 2006).

An additional pathway available for the transcellular transport of proteins is the receptor-mediated endocytosis, wherein, the protein molecules bind-specific receptors presented on the cell surface (Morishita et al., 2002; Peppas and Donini, 2006). The ligand-receptor complex is then endocytosed into the cells by the process of receptor-mediated endocytosis. The transport of molecules via this pathway is considerably faster than the passive diffusion pathway (Peppas, 2006; Wood et al., 2006a,b,c).

Insulin receptors have been identified in the basolateral membranes of dog intestinal mucosa, in the mouse intestinal cells and in the membrane of Caco-2 cells (Gingerich et al., 1987; Gallo-Payet and Hugon, 1984; Pillion et al., 1985; Torres-Lugo and Peppas, 2000). The precise role of these receptors in the GI tract remains unclear. Kendzierski et al. (2000) analyzed the ability of the gut to make insulin. It was suggested that the insulin receptors might be involved in an autocrine or paracrine role of the insulin made in the gut. Intracellular immunoreactivity towards insulin was found in glandular cells in the stomach and colon but no immunoreactivity was found in the small intestine (Saffran et al., 1997; López and Peppas, 2004a,b; Morishita et al., 2004; Peppas, 2004). In addition, the preproinsulin mRNA was detected in similar cells in the stomach and

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doi:10.1016/j.ijpharm.2007.12.014

colon. Insulin produced in the gut may function in controlling cell division, secretion of other peptides, or motility and absorption.

Importantly, insulin has been reported to be absorbed into the rat ileal epithelium in the presence of permeation enhancers and protease inhibitors (Bendayan et al., 1990; Ziv et al., 1987; Morishita et al., 1993; Blanchette and Peppas, 2005a,b, 2007). By applying protein A-gold immunocytochemical technique, Bendayan et al. (1990) showed that insulin was uptaken into endocytotic vesicles and was routed to the basolateral side of the ileal membrane via the trans-side of the Golgi-apparatus (Bendayan et al., 1994; Goto et al., 2006). A similar pathway of the insulin uptake and transport was also observed in normal and diabetic rats. In addition to this, an insulin-degrading enzyme (IDE), a thiol metalloprotease, has been identified in the cytosol of the rat intestinal enterocytes constituting up to 92% of total insulin-degrading activity (Bai and Chang, 1995). These results indicate that the ligand-specific receptor-mediated transcellular pathway may be functional in protein transport across the intestinal epithelium.

Thus both the paracellular and transcellular pathways could be functional in insulin transport across the intestinal epithelium. However, because of the endosomal degradation and the degradation due to the IDE, the contribution of the transcellular pathway to the insulin flux across the intestinal mucosa would be negligible (Bai and Chang, 1995; Nakamura et al., 2004; Foss and Peppas, 2004; Fisher et al., 2006; Fisher and Peppas, 2008).

The precise mechanism of insulin transport across the epithelium when delivered using the P(MAA-g-EG) microparticles is not clear (Torres-Lugo et al., 2002a,b). The polymeric microparticles have been shown to reversibly reduce the transepithelial electrical resistance (TEER) of the Caco-2 cell monolayer (Foss et al., 2004; Torres-Lugo and Peppas, 2002), which is a measure of the integrity of the tight junctions between the cells. This opening of the tight junctions by P(MAA-g-PEG) microparticles has been attributed to their ability to bind free calcium. Any significant decrease in calcium concentration of the medium surrounding the epithelial cells opens the tight junctions (Lacaz-Vieira, 1997; Sedar and Forte, 1994). This suggests that the microparticles facilitate the paracellular transport of insulin (Donini et al., 2002; Kavimandan et al., 2003, 2006a,b; Carr et al., 2007; Tuesca et al., 2008; Wood et al., 2008). It is, however, unclear whether this is the only mechanism by which insulin can be transported across the epithelium. Understanding the precise mechanism of enhancement in insulin transport in the presence polymeric microparticles is critical to improving the oral bioavailability of the protein (Ichikawa and Peppas, 2003; Besheer et al., 2006a,b; Peppas and Donini, 2007; Peppas, 2007).

In this study, the transport mechanism of fluorescein isothiocynate insulin (FITC-insulin) (Sigma, St. Louis, MO) across the Caco-2 cell monolayer in the presence of P(MAA-g-EG) microparticles was investigated by using confocal laser scanning microscopy. In order to distinguish between intracellular and paracellular insulin, the cells were labeled using Nile Red dye (Sigma, St. Louis, Mo), a lipophilic dye used to label the cell membranes.

2. Materials and methods

2.1. Preparation of polymer microparticles

P(MAA-g-EG) hydrogels were prepared by free radical solution UV-polymerization of methacrylic acid (MAA) and poly(ethylene glycol) ether monomethacrylate (PEGMA). The monomers were mixed in the molar ratio of 1:1 (MAA:EG). The MAA (Aldrich Chemical Company, Milwaukee, WI) was vacuum distilled at 47 °C/25 mmHg to remove the inhibitor hydroquinone monomethyl ether. PEGMA (Polysciences Inc., Warrington, PA) was used as received.

PEGMA with PEG molecular weight of 1000 was used in this synthesis. Tetra(ethylene glycol) dimethacrylate (TEGDMA) (Polysciences Inc., Warrington, PA) was used as the crosslinking agent and was added in the amount of 0.75% moles of the total amount of monomers. The photoinitiator, 1-hydroxy-cyclohexl-phenylketone (Irgacure-184) (Ciba-Geigy Co., Hawthorne, NY), was added in the amount of 0.1 wt% of the total amount of monomers. To inhibit autoacceleration in the polymerization reaction, the monomer mixture was diluted with a mixture of 50% (v/v) ethanol and deionized water (Milli-Q Plus System, Millipore). Nitrogen was bubbled through the well-mixed solution for 15 min to remove dissolved oxygen, which acts as a free radical scavenger. The mixture was then carefully poured between microscope slides (75 mm × 50 mm × 1 mm) (Fisher, Pittsburgh, PA) separated by Teflon spacers with a thickness of 0.9 mm. The glass slides were then placed in a nitrogen atmosphere under a UV light source of 16 mW/cm^2 at 365 nm for 30 min.

After the completion of the reaction, the polymer films were washed in deionized water for approximately 7 days in order to remove unreacted monomers, crosslinking agent, initiator and sol fraction. After washing, the polymer films were dried at room temperature for a day and then placed in a vacuum oven at 27 °C for 2 days. The dry polymer films were then crushed by using a mortar and pestle and sieved to 150–212 μm. All the particles were stored in a closed 5 mL glass vials inside a desiccator until further use.

2.2. Development of Caco-2 cell monolayer

The cells were cultured in 75 cm^2 culturing flasks (VWR Scientific, West Chester, PA) with 10 mL of Dulbecco's Modified MEM, culture media, DMEM (Bio Fluids, Biosource International). The seeding density for cultivation was 2.5×10^5 cells/flask. Cells were maintained in an incubator at 37 °C temperature, 95% relative humidity, and 5% CO_2. The culture medium was replaced with fresh medium every other day for about 6 or 7 days, until the cells reached 70–80% confluence. A passage operation was performed after the cells reached 60–80% confluence. In the passage operation, the cells were detached from the culturing flask by trypsinization, counted and transferred with the desired seeding density to a new culturing flask or experimental wells. In these cells studies, cells with passage numbers between 60 and 65 were used.

For transport studies, Caco-2 cells were grown in six-well Transwell® plates (4.71 cm^2/well) (Costar Corning Inc., Corn-

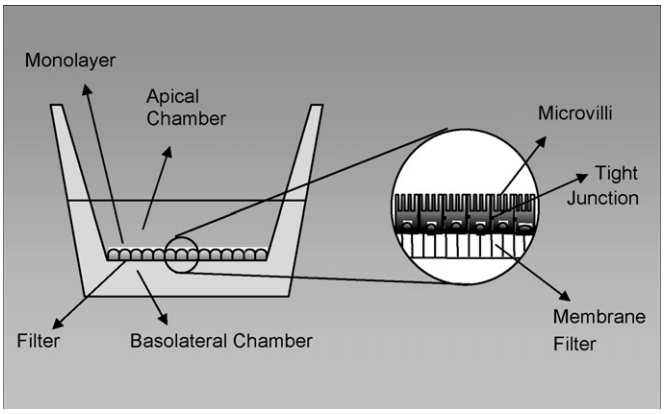

Fig. 1. Experimental setup for transport studies using Caco-2 cell monolayers. The cells were seeded on the Transwell plates and grown for 21 days to form fully differentiated cell monolayer.

ing, NY). The culturing cell density was 2.35×10^5 cells/well. The cells were grown in a DMEM culture media containing fetal bovine serum (FBS) for 21–24 days until they achieved a constant transepithelial electrical resistance, which indicated that the tight junctions had formed in the monolayer (Gumbiner, 1987; Denker and Nigam, 1998). The medium was changed every other day and the electrical resistance was monitored using a voltmeter with a chopstick electrode (World Precision Instrument, Sarasota, FL). The experimental setup is shown in Fig. 1. Each well consisted of two chambers: the apical (top) and the basolateral (bottom), which were separated by membrane with 3.0 μm pore size. The medium used for these studies was Hanks' balanced salt solution, HBSS containing Ca^{2+}(Peppas and Kavimandan, 2006; Yamagata et al., 2006)

2.3. *Confocal microscopy analysis of FITC-insulin transport*

Cell membranes were allowed to equilibrate for 1 h with the experimental medium, HBSS with Ca^{2+}, prior to the initiation of the experiment. HBSS containing Ca^{2+} was used since the absence of Ca^{2+} from the medium itself can cause opening of the tight junctions. After this period, the cell monolayer achieved a constant electrical resistance after the change of the medium.

The culturing medium from the six wells was removed and substituted by HBSS-containing FITC-insulin with or without pre-swollen microspheres. The wells were then incubated for 120 min and Nile Red dye (Sigma, St. Louis, MO) was applied on the apical and the basolateral sides of each well. The final concentration of Nile Red dye was 5 μL/mL. The cells were incubated for 5 min for labeling the membranes. The experimental medium was then removed and the membranes were gently rinsed with fresh media. The cells were fixed by incubation with 3.7% formaldehyde in Dulbecco's modified Phosphate Buffer Saline, DPBS modified (VWR Scientific, West Chester, PA) for 10 min. After fixation, the formaldehyde solution was removed and cells were gently washed with HBSS. The membrane was removed from the plastic insert by cutting it with a scalpel. It was then placed on a microscope slide, covered with a cover slip and placed under the microscope. The equipment consisted of a MRC 1024 with a Nikkon Diaphot 300 (BioRad, Hercules, CA).

3. Results and discussion

Confocal laser scanning microscopy experiments were performed to visualize the transport of FITC-insulin across Caco-2 cell monolayer in the presence or in the absence of P(MAA-g-EG) microparticles. Nile Red dye was used to label the membrane in order to clearly distinguish between paracellular and transcellular insulin in the images.

Figs. 2–3 illustrate the results obtained from confocal laser scanning microscope. Each figure is presents two X–Y images, the first one showing FITC-insulin (green) and Nile Red dye (red) and the second one showing the transmission image. Fig. 2 shows a control Caco-2 cell monolayer (not incubated with FITC-insulin) labeled with the Nile Red dye. The dye success-

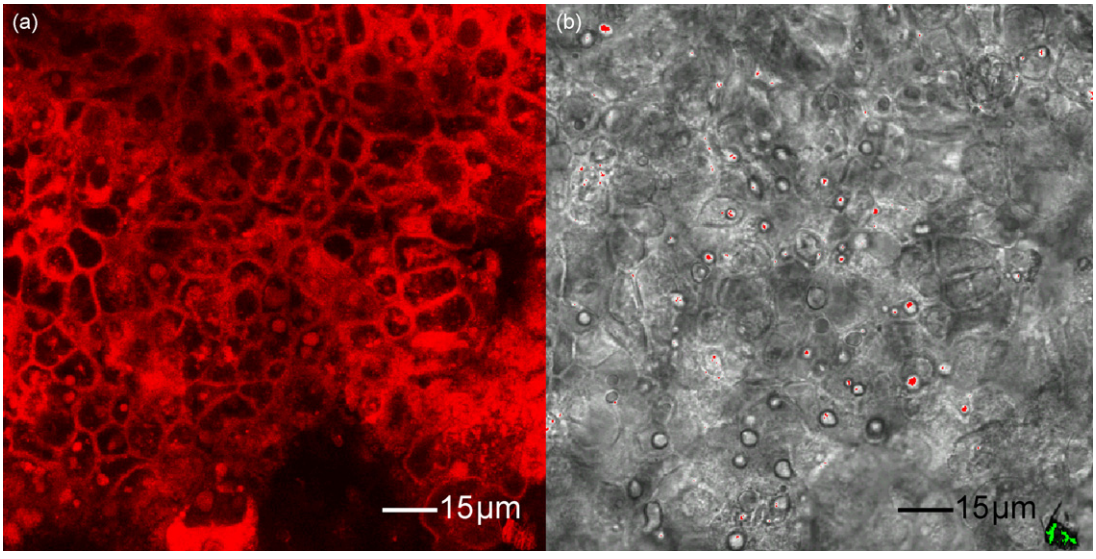

Fig. 2. Confocal laser scanning microscopy images of cells incubated with Nile Red dye. The combination of images (X–Y sections) illustrates (a) Nile Red dye (red) labeling the cells, (b) transmission image. The Nile Red dye is seen labeling the paracellular spaces but the dye can also be seen entering the cells.

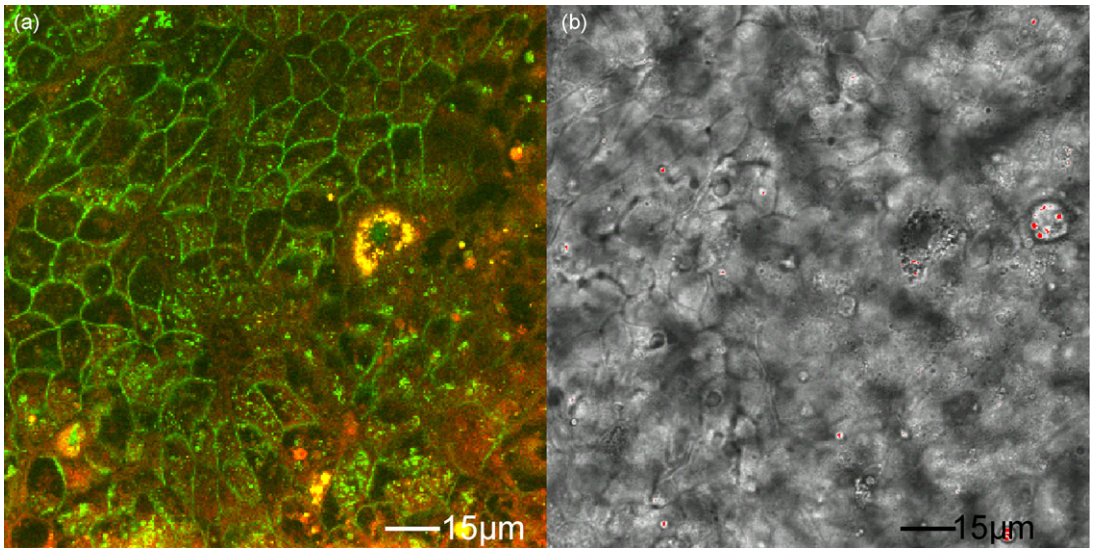

Fig. 3. Confocal laser scanning microscopy images cell monolayers with FITC-insulin. The cells were incubated with FITC-insulin without microsphere suspension. The cell monolayer was labeled with Nile Red dye. The combination of images (X–Y sections) illustrates (a) Nile Red dye (red) and FITC-insulin (green) co-labeling the cells, (b) transmission image. The enclosed spaces indicate insulin entering the paracellular spaces and the green dots inside the cells represent insulin uptaken by the cells.

fully labeled the cell membranes, although some dye diffused inside the cells. The images are not very clear, primarily because high concentration of the labeling solution was used and the washing process after the labeling was not efficient in removing the excess dye. Based on this observation, lower concentration of the labeling solution was used to label the cells in the subsequent experiments. This experiment established the ability of Nile Red dye to stain the cell membrane of Caco-2 cells in a confluent monolayer (Morishita et al., 2006a,b).

Fig. 3 shows confocal laser scanning microscope images of FITC-insulin placed on the basolateral side of cell membrane without any microparticles. Some important conclusions can be drawn from these images. The examination of images indicated that FITC-insulin present in the intercellular spaces can itself be used to visualize the cell membranes. Hence, the need for a lipophilic dye such as the Nile Dye in order to distinguish between the paracellular and transcellular insulin was eliminated. The images also revealed that some FITC-insulin was present inside the cells.

This insulin can be seen in the form of green dots localized in the intracellular spaces. Such green dots were seen predominantly in the X–Y sections near the apical and the basolateral sides of cell monolayer. As discussed in Section 1, the insulin receptors present on the cells could be functional in absorbing the labeled insulin into the cells. However, no insulin was seen in the transcellular spaces in the optical sections near the baso-

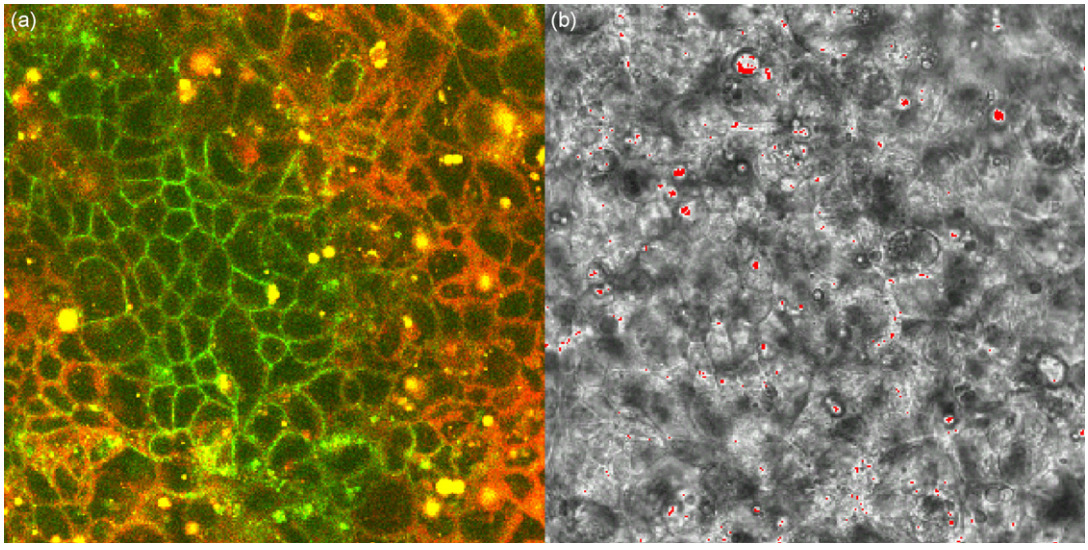

Fig. 4. Confocal laser scanning microscopy images cell monolayers with FITC-insulin and microparticles. The cells were incubated with FITC-insulin with microsphere suspension and labeled with Nile Red dye. The combination of images (X–Y sections) illustrates (a) Nile Red dye (red) and FITC-insulin (green) co-labeling the cells, (b) transmission image.

lateral membrane. This indicates that the insulin that entered the cells via the paracellular route was not able to reach the basolateral side. This could be either because of the degradation of protein in the endosomal vesicles or due to the degradation by the insulin-degrading enzyme. Hence the transcellular component of the insulin transport is either extremely low or entirely absent (Peppas et al., 2006; Kavimandan et al., 2006a,b).

Fig. 4 shows images of FITC-insulin placed on the basolateral side of the cell membrane with microparticles. The FITC-insulin was again located in the paracellular spaces. The intensity of fluorescence was only slightly higher as compared to the membrane incubated with FITC-insulin without any microparticles. Probable cause of this increase in the intensity is enhancement in the paracellular transport of insulin because of the presence of the microparticles. Interestingly, the green dots indicating transcellular insulin were not so prominent in these monolayers compared to the monolayer incubated with FITC-insulin only. The reasons behind the absence of FITC-insulin localized in the transcellular were not clear. This could be because clear images were not obtained with microparticles for focal planes near the apical side since emission due to excess concentration of Nile Dye was picked up in the channel for FITC-insulin. This made it difficult to analyze the optical planes near the apical region for the signal from transcellular FITC-insulin. This excess emission, also called bleed over, can also be noticed in Fig. 4.

4. Conclusions

Confocal microscopy studies were performed to evaluate the transport mechanisms of insulin across the Caco-2 cell monolayers. It was shown that the paracellular pathway was the predominant route of insulin transport across the monolayers in the presence or absence of the microparticles. The transcellular component for insulin transport was almost entirely absent even though uptake of insulin by the cells was observed. This is an important consideration in employing strategies to increase the flux of insulin across the Caco-2 cell monolayers and across the intestinal epithelium. It is suggested that insulin-degrading enzyme inhibitors such as *N*-ethyl maleimide, 1,10-phenanthroline, and EDTA may be used to achieve improved insulin absorption in the intestine (Morishita and Peppas, 2006). However, such inhibitors may have adverse effect on the functioning of the intestinal enzymes and may also cause permanent damage to the epithelium. Based on the observations from the confocal microscopy investigations presented here, we targeted transcellular pathway for improving insulin permeability.

Acknowledgements

This work is dedicated to Professor Tsuneji Nagai of Hoshi University for his exceptional and far reaching contributions to pharmaceutical sciences. Both of us have worked with Professor Nagai and the junior author (NK) was a student in his laboratories in Tokyo 3 years ago. The senior author (NAP) has been working with Dr. Nagai since 1985, having met him in scientific meetings in 1981. He has learned from his scientific enthusiasm and pioneering work and has been particularly indebted to him and his group for their significant contributions to oral protein delivery systems, where professor Nagai and several of his associates were co-authors in the very early papers of this laboratory.

This work was supported by a grant from the National Institutes of Health EG-000246.

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