Tyrphostin-8 Enhances Transferrin Receptor-Mediated Transcytosis in Caco-2 Cells and Increases Hypoglycemic Effect of Orally Administered Insulin-Transferrin Conjugate in Diabetic Rats

Cindy Q. Xia[1] and Wei-Chiang Shen[1,2]

Received August 22, 2000; accepted November 2, 2000

Purpose. To investigate the effect of tyrphostin 8 (T-8), a GTPase inhibitor, on transferrin receptor (TfR)-mediated transcytosis of insulin-transferrin (In-Tf) conjugate in cultured enterocyte-like Caco-2 cells and on gastrointestinal (GI) absorption of In-Tf in streptozotocin (STZ)-induced diabetic rats.

Methods. Caco-2 cells and diabetic rats were used as *in vitro* and *in vivo* models, respectively. TfR-mediated transcytosis was measured using ^{125}I-In-Tf. The absorption of insulin in diabetic rats was demonstrated by the hypoglycemic effect. Rat blood glucose level was determined using a ONE TOUCH® blood glucose monitoring system.

Results. T-8 increased apical-to-basolateral transport of In-Tf conjugate by enhancing TfR-mediated transcytosis in filter-grown Caco-2 cell monolayer, and this enhancement was higher and faster than the previously reported brefeldin A (BFA)-induced effect. The measurement of transepithelial electrical resistance (TEER) during the transport study showed that T-8 was less destructive on the cell tight junction than BFA. The GI absorption of In-Tf was evaluated by its hypoglycemic effect after oral administration in STZ-induced diabetic rats. The glucose-lowering effect of orally administered In-Tf in STZ-induced diabetic rats was improved by either T-8 or BFA. However, the effect of T-8 was more potent than that of BFA, especially at 7 h after administration. Either non-conjugated insulin or insulin-human serum albumin (In-HSA) conjugate by itself or in combination with T-8 did not show any hypoglycemic effect after oral administration, indicating that T-8-enhanced hypoglycemic activity of In-Tf was due to a selective enhancement of TfR-mediated transcytosis.

Conclusions. Our data indicated that T-8 could be used to increase the GI absorption of insulin as a transferrin conjugate. T-8, as an enhancer of TfR-mediated transcytosis, is better than the previously reported BFA. T-8 produces a higher increase on the transport of In-Tf and a lower toxicity on epithelial cells. Our findings provide an alternative approach to promote the GI absorption of insulin, as well as other peptide or protein drugs.

KEY WORDS: tyrphostin 8; In-Tf conjugate; oral delivery; Caco-2 cells; diabetic rats.

[1] Department of Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, California 90089-9121.

[2] To whom correspondence should be addressed. (e-mail: weishen@hsc.usc.edu)

ABBREVIATIONS: BFA, Brefeldin A; Caco-2, a human colon adenocarcinoma cell line; HSA, human serum albumin; In-HSA, insulin-human serum albumin conjugate; In-Tf, insulin-transferrin conjugate; i.p., intraperitoneal; PBS, phosphate-buffered saline; STZ, streptozotocin; TEER, transepithelial electrical resistance; Tf, transferrin; T-8, Tyrphostin 8.

INTRODUCTION

Over the last decade, transferrin receptor (TfR) has emerged as a tool that can be used as a marker for drug targeting or as a vehicle for transcytotic drug delivery (1). Using TfR as a target and transferrin (Tf) as a vector for transcellular transport is a novel way of achieving selective oral delivery of protein or peptide drug such as insulin across the intestinal epithelium (2). Although Tf has many promising features that make it a potential receptor-mediated drug carrier for transepithelial transport in gastrointestinal (GI) tract, there are limitations which stand in the way of an efficient transcytotic delivery of proteins. The foremost limitation of TfR-mediated transepithelial delivery system is that the rate of transport is very low because of the polarized distribution of receptors in the apical and basolateral plasma membranes of the intestinal epithelial cells. This is disadvantageous for the oral delivery of insulin via TfR-mediated transcytosis where the transport has to occur from the apical (i.e. mucosal) to the basolateral (i.e. serosal) side. Therefore, it is conceivable that an enhancement of TfR-mediated transcytosis will markedly improve the GI absorption of proteins as Tf conjugates.

Brefeldin A (BFA), a fungal metabolite, has been demonstrated to be an effective enhancer for TfR-mediated transcytosis in MDCK and Caco-2 cells (3,4), as well as for the oral absorption of insulin-transferrin (In-Tf) conjugate in streptozotocin (STZ)-induced diabetic CF/1 mice (5). However, the enhancement of TfR-mediated transcytosis of In-Tf in Caco-2 cells by BFA is only 3-fold over the control (4). In addition, BFA has not been used as drug in humans, and the long-term side effects are largely unknown. However, it is known that BFA can exert its biologic effects at several intracellular pathways (6). Therefore, it would be desirable to identify other potential enhancers with a well-defined action and a higher efficiency.

Recently, it has been reported that small GTPases such as Rab and Rho family molecules were involved in TfR-mediated endocytosis and transcytosis (7,8). Therefore, GTPase inhibitors that interfere with key GTPases, such as Rab4 or Rab11, could possibly control the sorting of membrane proteins from basolateral endosomes to either TGN or late recycling endosomes. In this report, we demonstrate that tyrphostin 8 (T-8, 4-hydroxybenzylidene-malononitrile), a known GTPase inhibitor (9), can increase intestinal epithelial transport of In-Tf conjugate in both Caco-2 cells and the GI of STZ-induced diabetic rats by enhancing TfR-mediated transcytosis. Our findings reveal that the effect of T-8 on the transcytosis of In-Tf conjugate was not only more potent, but also less toxic to epithelial cells than that of BFA. T-8 can potentially be developed into an efficient and specific enhancer for the GI absorption of protein and peptide drugs via TfR-mediated transcytosis.

MATERIALS AND METHODS

Materials

Cell culture medium and reagents were purchased from Gibco BRL® (Rockville, MD). N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and Iodo beads were ob-

0724-8741/01/0200-0191$19.50/0 © 2001 Plenum Publishing Corporation

tained from Pierce Chemical Company (Rockford, IL). All other chemicals not specified above were purchased from Sigma (St. Louis, MO).

Preparation of In-Tf or Insulin-Human Serum Albumin (In-HSA) Conjugate

In-Tf conjugate was prepared by the procedure previously described (2; 4). In-HSA was synthesized using the same method except that Tf was replaced by human serum albumin (HSA).

Caco-2 Cell Culture

Caco-2 cells (American Type Culture Collection, Rockville, MD; passage number 20) were grown on 0.4 μ pore size polycarbonate filters in Transwells (Costar, Cambridge, MA) as previously described (10). The cell monolayers (passage 20–40) were used for experiment 7 days after confluence to ensure that there was a complete differentiation into enterocyte-like cells. At that time, the transepithelial electrical resistance (TEER), measured by an epithelial voltohmmeter (EVOM, World Precision Instruments, West Haven, CT), was about 500 ohms · cm^2.

Apical-to-Basolateral Transcytosis of ^{125}I-Insulin, ^{125}I-In-Tf, and ^{125}I-In-HSA in Caco-2 Cell Monolayers

Recombinant human insulin, In-Tf and In-HSA were iodinated using the Iodo-beads method as suggested by the supplier, Pierce Chemical Company. Cell monolayers were washed once with DMEM at room temperature, and then incubated at 37°C for 1 h with DMEM containing 0.1% bovine serum albumin (BSA) to deplete serum Tf. Subsequently, ^{125}I-In-Tf conjugate (1.5 μg/ml) was added with or without a 100-fold excess of unlabeled Tf to the apical (1.5 ml) medium of filter-growth Caco-2 cells. In the test wells, ^{125}I-In-HSA or ^{125}I-insulin (at the same insulin concentration as that in the In-Tf conjugate) with or without a 100-fold excess of unlabeled insulin was added to the apical media. The enhancer (500 μM T-8 or 1.6 μg/ml BFA) was then added to the apical medium of certain Transwells. At various time intervals, the basal media containing transcytosed ^{125}I-In-Tf, ^{125}I-In-HSA, or ^{125}I-In were collected and replenished with an equal volume of fresh media. The collected samples were subject to 15% trichloroacetic acid (TCA) precipitation, and the radioactivity level in the protein precipitate was measured by using a Packard gamma counter. Receptor-mediated transcytosis was calculated by subtracting non-specific transport (with unlabeled Tf or insulin) from the total transport (without unlabeled Tf or insulin). The integrity of the cell monolayers was monitored during the experiment by measuring TEER using an epithelial voltohmmeter.

Diabetic Animal Model

Sprague-Dawley female rats (HARLAN, San Diego, CA) weighing 220–240 g were infected with freshly prepared streptozotocin (STZ, in pH 4.5 acetate buffer) by i.p. Five days after STZ-treatment, the fasted diabetic rats with a plasma glucose level >300 mg/dl were selected for the following investigation.

Animal Studies

The diabetic rats were fasted for 12 h and were then orally administered with insulin, In-Tf conjugate, In-HSA conjugate, or placebo (PBS) together with or without BFA (4.25 mg/kg) or various concentration of T-8 in sodium bicarbonate ($NaHCO_3$, 30 mg/ml) solution by using a gavage needle. The formulation with $NaHCO_3$ was used to neutralize the stomach acid and protect insulin, In-Tf or In-HSA conjugate from gastric digestion. The dosed rats were kept in metabolic cages with free access to water. The rats were fed with excess food immediately after the experiment was terminated. The glucose level of each animal was assessed before treatment, and a blood sample was periodically taken from the tail vein after oral drug treatment. The blood glucose levels were determined using a ONE TOUCH® blood glucose monitoring system (Lifescan Inc., Milpitas, CA), and were expressed as the percent change of each initial level. The absorption of insulin was indicated by the hypoglycemic effect.

RESULTS

Apical-to-Basolateral Transcytosis of the Conjugate Across Filter-Grown Caco-2 Cells

We previously reported that In-Tf could increase the transepithelial transport of insulin via TfR-mediated transcytosis, and that BFA could enhance this transcytosis in Caco-2 cells (4). In this study, we investigated the effect of T-8 on the apical-to-basolateral transcytosis of In-Tf conjugate in Caco-2 cells, and compared the result with that of BFA. As Figure 1 shows, In-Tf, but not In-HSA, increased insulin transcytosis. Furthermore, T-8 (500 μM) increased the specific

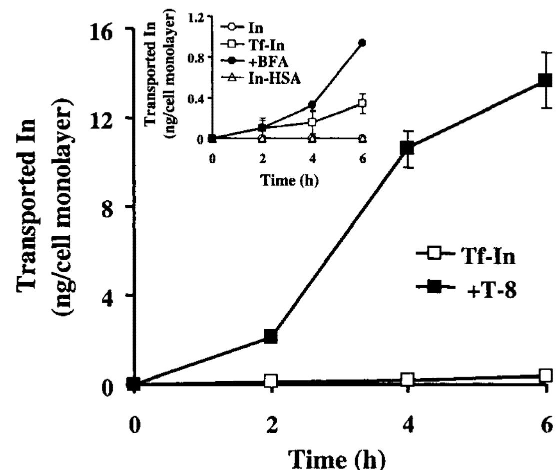

Fig. 1. Specific Apical-to-basolateral transcytosis of In-Tf conjugate in Caco-2 cells. ^{125}I-In-Tf conjugate, ^{125}I-In-HSA, or ^{125}I-insulin, together with or without BFA (1.6 μg/ml) or T-8 (500 μM) was added to the apical medium of 14-day cultured Caco-2 cells in Transwells. Cells were incubated with the medium at 37°C for various time intervals, and the medium containing the transcytosed conjugate or transcytosed insulin was collected. Specific transport was calculated by subtracting non-specific transport (with unlabeled Tf or insulin) from the total transport (without unlabeled Tf or insulin). The collected samples were subjected to 15% TCA precipitation, and the radioactivity associated with protein pellet was measured. Each point represents the mean ± s.d. (n = 3).

transcytosis of In-Tf conjugate about 20-fold, while BFA (1.6 μg/ml) only increased 3-fold in filter-grown Caco-2 cells. In-HSA was chosen as a control for the In-Tf conjugate because the molecular size of HSA (68 kDa) is similar to Tf (80 kDa).

The cell integrity was examined by measuring TEER during the transport study (Fig. 2). The TEER of Caco-2 cells was decreased 4 h after BFA treatment, but it was not affected by T-8 within 6-h treatment.

Hypoglycemic Effects of Oral Administration of In-Tf Conjugate with T-8

Both BFA and T-8 increased the GI absorption of In-Tf conjugate in STZ-induced diabetic rats which was indicated by the enhanced hypoglycemic effect (Fig. 3). T-8 seemed to enhance the absorption of In-Tf conjugate faster than BFA, especially at the 7th after drug-treatment. However, the end-points of blood glucose levels of the diabetic rats treated by In-Tf, In-Tf with T-8, or In-Tf with BFA were almost same, i.e. −80%, after 9 h treatment (Fig. 3). On the other hand, oral administration of insulin with T-8 did now show any significant change of the blood glucose level in diabetic rats (Fig. 3). Furthermore, the enhancement of T-8 on the absorption of In-Tf conjugate in STZ-induced diabetic rats was in a dose dependent manner (Fig. 4). In-Tf (equivalent to 80 U insulin/kg) itself could gradually decrease the glucose level in diabetic rats to about −50% of the initial level at 11th h after oral administration. On the other hand, the same amount of In-Tf with 12.75 mg/kg T-8 conjugate could decrease the glucose level to −74% at the 11th hour (122 ± 42 mg/dl (n = 3)), which was close to the fasted glucose level in normal fasted rats, i.e. 50 ± 7 mg/dl (n = 14) (Fig. 4). T-8 itself did not present any hypoglycemic effects (Fig. 4). All the rats regained the initial high glucose levels at 10 h after experiment termination (data not shown). No severe hypoglycemia was observed in these experimental rats.

Compared to the In-Tf (27 U insulin/kg) conjugate, In-HSA (27 U insulin/kg) conjugate with T-8 did not significantly change the blood glucose level after oral administration in STZ-induced diabetic rats. When combining either conjugate with 4.25 mg/kg of T-8, the glucose level decreased to 60 ± 12% of the initial glucose level at 11 h for In-Tf, while In-HSA showed no effect on blood glucose level (108 ± 14% of the initial glucose level).

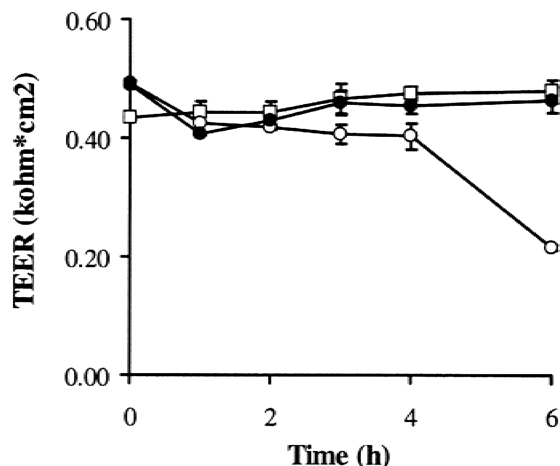

Fig. 2. TEER change in Caco-2 cells during transport study. The TEER was measured with an epithelial voltohmmeter at indicated time. Control (□) means the cell monolayer without any enhancer treatment. The doses of T-8 (●) and BFA (○) were 500 μM and 1.6 μg, respectively. Each point represents the mean ± s.d. (n = 6).

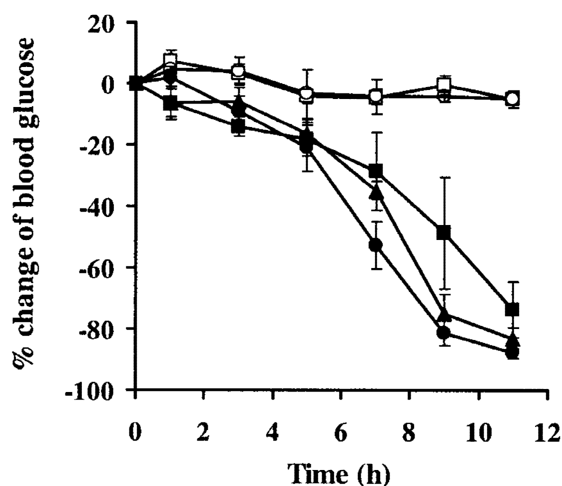

Fig. 3. Comparison of the effect of T-8 with that of BFA on oral absorption of In-Tf in STZ-induced diabetic rats. Placebo was PBS (□) and dosage of insulin or In-Tf was 80 U/kg. T-8 or BFA (both 4.25 mg/kg) was used in the study. Insulin together with T-8 (○) was used as a control to investigate the effect of T-8 on insulin function. The blood glucose level of the diabetic rats treated by In-Tf alone (■) or with T-8 (●) or BFA (▲) was compared. The initial blood glucose levels of diabetic rats treated by PBS, insulin with T-8, In-Tf with T-8 and In-Tf with BFA were 340 ± 39, 371 ± 10, 324 ± 14, and 330 ± 4, respectively. Results are expressed as the mean ± S.E.M. (n = 3, 4, t-test for In-Tf conjugate with BFA or T-8: $p < 0.10$).

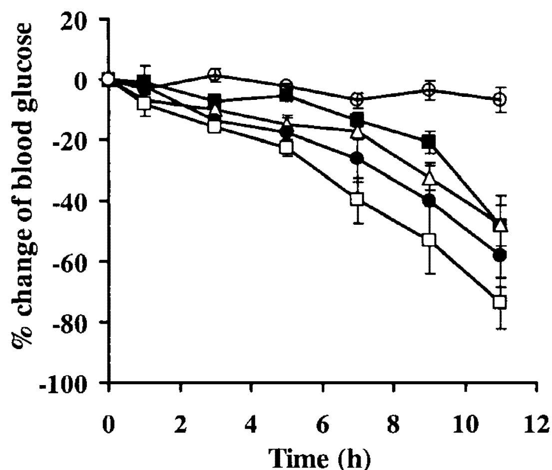

Fig. 4. Changes in blood glucose levels of STZ-induced diabetic rats after orally administered In-Tf conjugate with different concentration of T-8. In-Tf (equivalent to 80 U/kg of insulin) with 0 (■), 1.42 (△), 4.25 (●), or 12.75 (□) mg/kg of T-8, was orally given to the STZ-induced diabetic rats. T-8 (12.75 mg/kg) alone was used as control (○). The initial blood glucose levels of the diabetic rats treated with 12.75 mg/kg T-8 alone, and 0, 1.42, 4.25, and 12.75 mg/kg T-8 with In-Tf were 422 ± 7, 401 ± 10, 450 ± 25, 416 ± 22, and 433 ± 35, respectively. Results are expressed as the mean ± S.E.M. (n = 3, 4; t-test for In-Tf with or without 1.42 mg/kg T-8: $p < 0.05$; t-test for In-Tf with or without 4.25 mg/kg T-8: $p < 0.05$; t-test for In-Tf with or without 12.75 mg/kg T-8: $p < 0.01$.)

DISCUSSION

One limitation in developing Tf as a carrier for oral delivery of peptide or protein drugs is that the rate of TfR-mediated transcytosis in intestinal epithelial cells is generally very low. This shortcoming is most likely due to the polarity of TfR distribution which is predominate on the basolateral surface in intestinal epithelial cells (11). Therefore, the majority of membrane-associated TfR enters the cells via an endocytosis-recycling pathway at the basolateral membrane (12). The basolateral localization of TfR makes the orally administered Tf in the lumen of the GI tract inaccessible to the receptors on epithelial cells.

However, we observed recently that In-Tf could be absorbed as an intact conjugate and exhibit a slow but prolonged hypoglycemic effect via oral administration (2,13). In those studies, a relatively high dose of the insulin conjugate was administered in order to reduce blood glucose in diabetic rats to the normal level (2). The requirement of a high dose of In-Tf indicates that the GI absorption of this conjugate is inefficient. It is conceivable that the dose of In-Tf in oral administration would be markedly reduced if a specific enhancer could be utilized to increase the efficiency of TfR-mediated transcytosis in enterocytes.

We reported previously that BFA could enhance TfR-mediated transcytosis in cultured epithelial cells (3,14). This increase was highly reversible, specific to TfR (15), and appeared to be effective in most types of epithelial cells including enterocyte-like Caco-2 (14), kidney epithelial MDCK (3), and primary lung epithelial (16) cells. In addition to its effectiveness in many cell types, BFA is also known to interfere with functions of many intracellular organelles such as endosomes, lysosomes, endoplasmic reticulum, cis-Golgi cisterni, and trans-Golgi network (TGN) (17,18). Although BFA could increase TfR-mediated transcytosis to almost 100-fold in MDCK cells (3), it only produced a 3 to 5-fold enhancement in the enterocyte-like Caco-2 cells, compared to control (14). Due to the potential side effects on other epithelial cells and other intracellular organelles, and the low level of enhancement in Caco-2 cells, BFA is not an ideal agent for developing a therapeutically useful enhancer for the GI absorption of Tf-conjugated peptide and protein drugs. Therefore, our recent efforts have been focused on identifying other compounds that are not only more potent than BFA, but also more specific to TfR-mediated transcytosis and more selective to intestinal epithelial cells.

Data presented in this report suggest that T-8 is an effective enhancer for increasing the GI absorption of In-Tf. The magnitude of the enhancement on TfR-mediated transcytosis in Caco-2 cells is higher with T-8 than with BFA (Fig. 1). On the other hand, T-8 appeared to be less effective than BFA on disrupting tight junctions in Caco-2 cells (Fig. 2), suggesting that T-8 is less toxic than BFA to the GI epithelium. Furthermore, the enhancing effect of T-8 on TfR-mediated transcytosis was found only in Caco-2, but not in MDCK, cells (19), suggesting that it may be more selective to enterocytes than other types of epithelial cells. Therefore, we conclude that T-8 is a better candidate for enhancing GI absorption of peptide or protein drugs via TfR-mediated transcytotic pathway.

Results from *in vivo* experiments of treating diabetic rats with T-8 and In-Tf qualitatively confirmed the cell culture studies. The hypoglycemic response to oral administered In-Tf in diabetic rats was more effective in the presence of T-8 than of BFA which, in turn, was more effective than of In-Tf alone. As shown in Fig. 3, the onset of the hypoglycemic effect of orally administered In-Tf in T-8 treated rats occurred sooner than in BFA-treated and control rats. The blood glucose level in T-8 treated rats was also lower than in BFA-treated or control rats (Fig. 3). However, the magnitude of the enhanced hypoglycemic effect with either T-8 or BFA treatment (Fig. 3) was much less impressive than that of the enhanced TfR-mediated transcytosis observed in cultured Caco-2 cells (Fig. 1). This discrepancy could be attributed to several factors. First, in cell culture studies, the dose of T-8 was 50-fold higher by mass than that of BFA, while in animal experiments the dose was 4.25 mg/kg for both agents. It is possible that a higher dose of T-8 is required in order to achieve a maximal effect. This assumption is consistent with the finding that the effect of T-8 on TfR-mediated transport of In-Tf was dose-dependent in both cultured Caco-2 cells (20) and in diabetic rats (Fig. 4). Second, it is conceivable that BFA, a palmitic acid lactone derivative, is more lipophilic than T-8, and is absorbed more rapidly and metabolized more slowly than T-8 in the GI tract. Therefore, the net amount of T-8 absorbed by the rats may be less than that of BFA, although an identical dose of BFA and T-8 was used in the animal experiments. Without a complete pharmacokinetic profile, it is difficult to determine the doses of BFA and T-8 for a quantitative comparison of their enhancing effect on In-Tf absorption in animal models.

Another possibility of the quantitative difference between the measurement of transport in cultured cells and the determination of blood glucose levels in diabetic rats is that the rate-limiting step for the hypoglycemic effect could be the releasing of active insulin from In-Tf rather than the transport of In-Tf across enterocytes. This assumption is consistent with the observation that orally administered In-Tf, either with or without enhancers, has never produced a severe hypoglycemia in rats. In both T-8 and BFA-enhanced hypoglycemic effects (Figs. 3 and 4), the blood glucose concentration appeared to stabilize at 10% of the initial level at 9 h after the administration. This level of blood glucose, approximately 30 to 40 mg/dl, is close to the blood glucose level in normal rats.

Therefore, in our experiments, none of the treated rats was in a risk of severe hypoglycemia, a serious side effect for hyperinsulinemia (21). Similar observations have been observed in both oral and subcutaneous administration with high doses of In-Tf without using enhancers (2). Since neither insulin (Fig. 3) nor In-HSA conjugate showed any hypoglycemic activity when administered orally with T-8, the observed In-Tf effect in the presence of T-8 must be due to a specific enhancement of TfR-mediated transcytosis of the intact conjugate. It is likely that the *in vivo* insulin-releasing rate from In-Tf is well controlled by a saturable rate-limiting step, and therefore, an over-dose of insulin can be avoided at high doses of In-Tf. This hypothesis is consistent with our previous finding that, 4 h after the oral administration of In-Tf, the intact conjugate rather than free insulin was found predominately in the plasma of the rats (2). Even though the rate-limiting step for releasing active insulin has not yet been identified, such a controlled insulin release from In-Tf in the blood, especially via oral administration, would be highly desirable for the treatment of diabetes.

ACKNOWLEDGMENTS

The work was supported by the grant from American Diabetes Association. The technical assistances of Mrs. Daisy Shen and Ms. Fariba Norouziyan are gratefully acknowledged.

REFERENCES

1. P. M. Friden. Receptor-mediated transport of peptides and proteins across the blood-brain barrier. In W. M. Pardrige. *The Brain Barrier,* Raven Press Ltd., New York, 1993 pp. 229–247.
2. C. Q. Xia, J. Wang, and W. C. Shen. Hypoglycemic effect of insulin-transferrin conjugate in streptozotocin-induced diabetic rats. *J. Pharmacol. Exp. Ther.* **295:**594–600 (2000).
3. J. Wan, M. Taub, D. Shah, and W. C. Shen. Brefeldin A enhances receptor-mediated transcytosis of transferrin in filter grown Madin-darby canine kidney cells. *J. Biol. Chem.* **267**:13446–13450 (1992).
4. D. Shah and W. C. Shen. Transepithelial delivery of an insulin-transferrin conjugate in enterocyte-like Caco-2 cells. *J. Pharm. Sci.* **85**:1306–1311 (1996).
5. I. Chantret, A. Barbat, M. G. Dussault, M. G. Brattain, and A. Zweibaum. Epithelial polarity, villin expression and enterocytic differentiation of cultured human colon carcinoma cells: A survey of twenty cell lines. *Cancer Res.* **48**:1936–1942 (1988).
6. R. D. Klausner, J. G. Donaldson, and J. Lippincott-Schuartz. Brefeldin A: Insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* **116**:1071–1080 (1992).
7. B. Sonnichsen, S. De Renzis, E. Nielsen, J. Rietdorf, and M. Zerial. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J. Cell Biol.* **149**:901–914 (2000).
8. S. M. Leung, W. G. Ruiz, and G. Apodaca. Sorting of membrane and fluid at the apical pole of polarized Madin-Darby canine kidney cells. *Mol. Biol. Cell.* **11**:2131–2150 (2000).
9. G. Wolbring, M. D. Hollenberg, and P. P. Schetkamp. Inhibition of GTP-utilizing enzymes by tyrphostins. *J. Biol. Chem.* **269**: 22470–22472 (1994).
10. E. J. Hughson, D. F. Cutler, and C. R. Hopkins. Basolateral secretion of kappa light chain in the polarized epithelial cell line, Caco-2. *J. Cell Sci.* **94(Pt2)**:327–332 (1989).
11. D. Banerjee, P. R. Flanagan, J. Cluett, and L. S. Valberg. Transferrin receptors in the human gastrointestinal tract. *Gastroenterology* **91**:861–869 (1986).
12. A. Dautry-Varsat. Receptor-mediated endocytosis: The intracellular journey of transferrin and its receptor. *Biochimie.* **68**:375–381 (1986).
13. J. Wang, D. Shen, and W. C. Shen. Oral delivery of insulin-transferrin conjugate in streptozotocin-treated CF/1 mice. *Pharm. Res. (Supplement)* **14**:469 (1997).
14. D. Shah and W. C. Shen. The establishment of polarity and enhanced transcytosis of transferrin receptors in enterocyte-like Caco-2 cells. *J. Drug Targeting* **2**:93–99 (1994).
15. W. C. Shen, D. Shah, M. Taub, and J. Wan. Enhancement of transferrin receptor-mediated transcytosis for transepithelial protein delivery. In V. H. L. Lee, M. Hashida, and Y. Mizushima (eds.), *Trends and future perspectives in peptide and protein drug delivery,* Harwood Academic Publishers, 1995 pp. 135–151.
16. D. Deshpande, D. Toledo-Velasquez, L. Y. Want, C. J. Malanga, J. K. Ma, and Y. Rojanasakul. Receptor-mediated peptide delivery in pulmonary epithelial monolayers. *Pharm. Res.* **11**:1121–1126 (1994).
17. J. Lippincott-Schuartz, L. Yuan, C. Tipper, M. Amherdt, L. Orci, and R. D. Klausner. Brefeldin A's effects on endosomes, lysosomes and TGN suggest a general mechanism for regulating organelle structure and membrane traffic. *Cell* **67**:601–616 (1991).
18. S. Wood, J. Park, and W. Brown. Brefeldin A causes a microtubule-mediated fusion of the TGN and early endosomes. *Cell* **67**: 591–600 (1998).
19. C. Q. Xia, F. Norouziyan, and W. C. Shen. Different effects of tyrphostin 8, a GTPase inhibitor, on transferrin receptor processing in MDCK and Caco-2 cells. *ASCB 40th Annual Meeting,* San Francisco, 2000.
20. C. Q. Xia and W. C. Shen. Tyrphostin 8: A new enhancer of transferrin receptor-mediated transcytosis in Caco-2 cells. *Proceed. Int'l Symp. Control. Rel. Bioact. Mater.* **26**:313–314 (1999).
21. R. A. Jackson, J. B. Hamling, P. M. Blix, B. M. Sim, M. I. Hawa, J. B. Jaspan, J. Belin, and J. D. Nabarro. The influence of graded hyperglycemia with and without physiological hyperinsulinemia on forearm glucose uptake and other metabolic responses in man. *J. Clin. Endocrinol. Metab.* **63**:594–604 (1986).