Pharmaceutical Research, Vol. 11, No. 12, 1994

Research Article

Insulin Fragments as a Carrier for Peptide Delivery Across the Blood–Brain Barrier

Makoto Fukuta,[1,2] Hiroaki Okada,[1,3] Satoshi Iinuma,[1] Shigeo Yanai,[1] and Hajime Toguchi[1,4]

Received February 12, 1994; accepted July 5, 1994

The possibility of using insulin (INS), which is transported into the brain by receptor-mediated transcytosis, as a peptide carrier for delivery across the blood-brain barrier (BBB) was investigated. After mice received an i.v. injection of horseradish peroxidase (HRP, M.W., 40,000) conjugated with INS, the HRP activity in the brain was higher than that after HRP injection. Since INS-HRP lowered the blood glucose level, we prepared insulin fragments by chemical and enzymatic procedures in an effort to find a carrier with no hypoglycemic activity. Seven fragments were synthesized taking the binding regions into consideration, but none showed any receptor binding affinity in cultures of bovine brain microvessel endothelial cells (BMEC). However, the fragment (F007) obtained by trypsin digestion showed high affinity and scarcely any hypoglycemic activity in mice even at a dose ten times the effective dose of insulin. These results suggest that this fragment may be useful as a carrier to transport therapeutic peptides across the BBB.

KEY WORDS: blood-brain barrier; horseradish peroxidase; receptor-mediated transport; insulin fragments; carrier for peptides; hypoglycemic activity.

INTRODUCTION

Peptides and proteins with CNS activity are potentially useful in the treatment of central nervous system diseases. These include vasopressin (1), β-endorphin (2) and nerve growth factor (NGF) (3,4). The size of these molecules however prevents them from easily passing through the blood-brain barrier (BBB) because of the tight junctions between the endothelial cells which act as a physical barrier. Therefore, it is necessary to develop a delivery system which can transport therapeutic peptides and proteins across the BBB. Several strategies to get peptides through the barrier have been tried. For instance, opening the barrier by carotid injections of hypertonic solutions (5) and inserting an intraventricular catheter through the skull (6) have been used successfully for delivery of enzymes and muscarinic agonist, respectively. However, these methods are invasive and probably not practical for use in large numbers of patients. Normal transport processes through the brain capillaries should be successful. For example, brain dopamine can be elevated in patients suffering from Parkinson's disease by administering it's precursor L-dopa (7) which is carried across the BBB by the carrier-mediated transport system for amino acids (8). Recent studies revealed that several peptides can be transferred into the brain by receptor-mediated and absorptive-mediated transcytosis (9,10). From the results of *in vitro* experiments using isolated brain capillaries or primary cultures of capillary endothelial cells, peptides in the circulation, such as insulin (INS), transferrin and insulin-like growth factor, are suggested to penetrate the BBB by receptor-mediated transcytosis (10,11). Thus, the receptor-mediated vector, OX-26 monoclonal antibody (murine monoclonal antibody against the rat transferrin receptor) (12) was successfully utilized to deliver the therapeutic peptides, such as NGF (13,14) and vasoactive intestinal polypeptide (VIP) (15) into the brain through the BBB. Substances such as cationized albumin or cationized immunoglobulin G can penetrate the BBB by absorptive-mediated transcytosis on the basis of the electrostatic interaction between the positively charged protein and negatively charged glycocalyx lining of the brain capillary endothelium (16,17). These studies on mechanisms of peptide transport across the BBB led to a new strategy for the delivery of peptides into the brain: using chimeric peptides of β-endorphin, a nontransportable peptide, covalently coupled to cationized albumin (18,19).

In the present study, we examined the usefulness of INS as a peptide and protein carrier for delivery to the brain using an INS-horseradish peroxidase (HRP) conjugate. Since the INS-HRP conjugate lowered the blood glucose level in mice, we then searched for insulin fragments which have no hypoglycemic effect but still have affinity for the insulin receptor using the *in vitro* cultured system of bovine brain microvessel endothelial cells (BMEC).

[1] DDS Research Laboratories, Takeda Chemical Industries, Ltd. 2-17-85 Jusohonmachi, Yodogawa, Osaka 532, Japan.
[2] Present Address: Pharmaceutical Development Laboratories, Takeda Chemical Industries, Ltd.
[3] To whom correspondence should be addressed.
[4] Present Address: Research on Research, Takeda Chemical Industries, Ltd.

0724-8741/94/1200-1681$07.00/0 © 1994 Plenum Publishing Corporation

MATERIALS AND METHODS

Materials

Chemicals were obtained from the following sources: [^{125}I]-Tyr^{A14} porcine insulin (2200 Ci/mmol) and [^{3}H]-inulin (10 mCi/g), New England Nuclear Corporation (NEN, Boston, MA); porcine insulin, Diosynth B.V. (BH Oss, Netherlands); N-succinimidyl 3-(2-pyridyldithio)proprionate (SPDP), Pierce Chemical Company (Rockford, IL); horseradish peroxidase (HRP), Wako Pure Chemical Industries Ltd. (Osaka, Japan); and trypsin, V8 protease and bovine serum albumin (BSA, RIA grade), Sigma Chemical Company (St. Louis, MO). All other chemicals were reagent grade.

Conjugate Synthesis

Ten milligrams of HRP was dissolved in 1 ml of 0.1 M phosphate buffer (pH 7.4) followed by the addition of 7 mg of SPDP dissolved in 40 μl dimethylsulfoxide (DMSO). Following incubation at room temperature (RT) for 30 min, HRP coupled with SPDP was separated from free SPDP using a PD-10 column (Pharmacia Biotech AB., Uppsala, Sweden) and eluting with the phosphate buffer. The first 2.8 ml eluted (Solution A) was collected. Also 7 mg of INS dissolved in 1 ml phosphate buffer was reacted with 7 mg of SPDP dissolved in 40 μl DMSO at RT for 30 min. INS coupled with SPDP was separated using a PD-10 column and eluting with 0.1 M acetate buffer (pH 4.5). The first 2.8 ml eluted was concentrated to 2 ml followed by the addition of 18 mg of dithiothreitol in 0.1 M acetate buffer (pH 4.5, 0.5 ml). Following incubation at RT for 30 min, INS coupled with SPDP was separated from dithiothreitol using a PD-10 column and eluting with the phosphate buffer, and the first 2.8 ml eluted (Solution B) was collected. Solutions A and B were mixed and incubated at RT for 18 hr. The INS covalently coupled with HRP was separated from HRP and INS using a 1.5 × 65 cm Sephadex G-75 column (Pharmacia Biotech AB.) and eluting with 0.05 M phosphate buffer (pH 7.4). Each 2 ml fraction was collected, tested to determine the absorbance at 280 nm and the HRP activity and analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The fractions showing HRP activity but not giving an HRP band upon SDS-PAGE were collected and dialyzed against 2 L of distilled water for 3 consecutive 8-hour periods using Visking tubing (Spectrapor, M.W. cut off: 12,000–14,000, Spectrum Medical Industries Inc., Los Angels, CA). After being freeze-dried, the yield of INS-HRP conjugate powder was 4.5 mg. INS-HRP conjugates had 65% of the activity of native HRP.

In Vivo Brain Uptake

Saline solutions (0.1 ml) containing 230 μg HRP or 213 μg INS-HRP conjugate were injected into male ICR mice (8–9 W, Charles Rivers Japan) via a tail vein. Blood and brains were collected after decapitation at appropriate intervals to determine the HRP activity. Blood samples treated with a little heparin were individually centrifuged at 3,000 g for 10 min to obtain plasma. Brains were individually homogenized in pH7.2, 20 mM phosphate buffered saline (PBS, 10 W/V% concentration) with a Polytron Homogenizer (KINEMATICA AG, Lucerne, Switzerland), and the homogenate was centrifuged at 25,000 g for 10 min. One hundred microliters of plasma diluted with PBS (×100) or supernatant of the homogenate diluted with PBS (×2) was mixed with 1 ml of a substrate solution (40 mM *o*-phenylenediamine in 0.1 M citrate buffer (pH 5.5) containing 0.066% hydrogen peroxide). The mixture was incubated at RT for 20–40 min in the dark, and 1 ml of 2 M H_2SO_4 was then added to stop the reaction. Thereafter, the absorbance at 492 nm was measured. The plasma values were corrected for blood distribution of HRP using the hematocrit value (0.42). To determine blood volume in the mouse brain, 0.1 ml of saline solution containing 1 μCi of [^{3}H]-inulin and 1 mg inulin was injected into mice in a satellite group (n = 5) via a tail vein. After 5 min, blood and brains were collected and their radioactivities were measured by a scintillation counter (Beckman Model 6800, Beckman Instruments, Fullerton, CA) after dissolution in SOLVABLE (NEN).

Preparation of Synthetic Insulin Fragments

Single chain insulin fragments (I-002: des A6, A1-8 insulin; I-003: B1-8, Cys^{7}(S-3-nitro-2-pyridinesulfenyl, Npys) insulin; I-005: B19-30, Cys^{19}(Npys) insulin; and I-006: B19-25 Cys^{19}(Npys), Tyr^{25}-amide insulin) were prepared by "efficient" solid phase peptide synthesis (20) using the *tert*-butoxycarbonyl (t-Boc) strategy. Amino acid loaded Merrifield resin or p-methylbenzhydrylamine resin (0.07–0.24 mmol, Watanabe Chemical Industrial Ltd, Hiroshima, Japan) was used as the starting material, and the following side chain protected t-Boc amino acids were used for synthesis: Cys(S-4-methylbenzyl), Cys(Npys), Glu(γ-benzyl), His(π-benzyloxymethyl), Arg(N-G-mesitylene-2-sulfonyl), Tyr(O-2-bromobenzyloxycarbonyl), Lys(N-ε-2-chlorobenzyloxycarbonyl) and Thr(O-benzyl). After constructing the desired peptide sequence, the protected resin was treated with hydrogen fluoride in the presence of m-cresol. All the protecting groups except for Cys(Npys) were removed during this treatment. The crude peptides were washed with dry ether and chromatographed on a 2.7 × 90 cm Sephadex G-25 column (Pharmacia Biotech AB.). The desired fractions were lyophilized. The double chain insulin fragments (I-004: des A6, A1-8, B1-8 insulin; I-007: des A6, A1-8, B19-30 insulin; and I-008: des A6, A1-8, B19-25, Tyr^{25}-amide insulin) were prepared by mixing I-002 and I-003, I-002 and I-005, and I-002 and I-006, respectively. The crude peptides were purified by reversed-phase HPLC (column: TSK gel ODS-120T, TOSOH, 5 μm, 2.15 ϕ × 30 cm) using a concentration gradient of acetonitrile containing 0.1% trifluoracetic acid. Each peptide was checked by amino acid analysis, purity was confirmed by analytical reversed-phase HPLC (column: μ Bondapak C_{18}, Waters, 15 μm, 3.9 ϕ × 150 mm) using a concentration gradient of acetonitrile containing 0.1% trifluoracetic acid.

Preparation of Insulin Fragments by Proteolitic Degradation

Three hundred milligrams of insulin was dissolved in 2.5 ml of 10 mM HCl and diluted with 150 ml of 0.1 M phosphate buffer (pH 7.4) followed by the addition of 45 mg trypsin. After incubation at 37°C for 36 hr, the reaction was stopped

by the addition of 10 ml of 1 N acetic acid and the solution was freeze-dried. The resulting powder (50 mg) was dissolved in 1 N acetic acid, and the three insulin fragments (F001, F006 and F007) were separated by reversed-phase HPLC (TSK-gel ODS-120T column, 2.15 ϕ × 30 cm) using a concentration gradient of acetonitrile containing 0.1% trifluoracetic acid. Also, 50 mg of insulin was dissolved in 1 ml of 10 mM HCl and diluted with 50 ml of 0.1 M phosphate buffer (pH 7.4) followed by the addition of 0.5 mg V8-protease. After incubation at 37°C for 48 hr, four insulin fragments (F002-F005) were collected by HPLC as described above.

Isolation and Culture of BMEC

Brain microvessel endothelial cells (BMEC) were isolated from the cerebral gray matter of the bovine brain as described by Audus and Borchardt (21–23). After isolation, approximately 1 × 10^5 cells/cm^2 were seeded on 22.6 mm diameter (4 cm^2) Costar culture dishes (Costar Corporation, Cambridge, MA) coated with rat collagen and bovine fibronectin. After 9–11 days in culture, they were examined by microscopy to determine the degree of confluency. The characteristics of the cultured BMEC are essentially identical to those of parent tissue (23).

Binding Affinity of Insulin and Insulin Fragments for BMEC

Prior to conducting the [^{125}I]-insulin binding assay, cell monolayers were washed two times with buffer A (120 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.05 mM NaEDTA, 1.2 mM NaH_2PO_4, 10 mM HEPES, pH 7.4) and then incubated at 37°C for 30 min in buffer A containing 0.1% BSA (buffer B). Total [^{125}I]-insulin binding was measured by exposing the cell monolayers to buffer B containing 0.025 nM [^{125}I]-insulin at 4°C for 60 min. The cells were then washed three times with fresh ice cold buffer A and removed from the dishes with two, 1 ml aliquots of 0.2 N NaOH. The cell suspension was analyzed for radioactivity (Beckman 5500 γ counter) and total cell protein measurement (DC PROTEIN Assay, Bio-Rad Laboratories, Hercules, CA). Competitive displacement experiments were conducted as described above with [^{125}I]-insulin (0.025 nM) except that a certain concentration of insulin, insulin fragments or INS-HRP was added to buffer B. The amounts of [^{125}I]-insulin bound to BMEC in the presence of the insulin, insulin fragments and INS-HRP was calculated as a percent of the maximum amount of [^{125}I]-insulin that was initially added to the cell monolayer.

RESULTS AND DISCUSSION

HRP was used as a model for nontransportable peptides and proteins because it is a water soluble large molecule (M.W. 40,000). Its ability to pass through the endothelial membrane of brain capillaries and into the brain parenchymal cells without degeneration was examined by measuring enzyme activity. Fig. 1 shows the percentage of the dose of HRP in the blood and brain of mice after injecting HRP and INS-HRP conjugate into a tail vein. Blood volume in the mouse brain was calculated to be 5.76±1.14 μl/g brain (mean±SE, n=5) from the radioactivity of [^{3}H]-inulin in the brain and blood 5 min after i.v. administration of [^{3}H]-inulin, which can hardly penetrate the BBB. The activity of HRP in 1 g of brain tissue after administration of intact HRP was not significantly different from that in 5.76 μl of blood. This indicates that HRP hardly penetrated into the brain. On the other hand, the HRP activity in 1 g of brain tissue after administration of HRP-INS conjugate was obviously higher than that in 5.76 μl of blood. This shows that INS-HRP conjugate having enzyme activity was efficiently taken up by the brain. To compare % of dose in the brain with that in the blood, the units on the axis of Fig. 1 are % of dose (per g of brain tissue, i.e. per 5.76 μl of blood). The brains in the mice used in this experiment weighed 0.405 g (mean, n=3). Therefore, INS-HRP conjugate including the dissociated HRP was taken up by the brain at 0.57% of the dose per brain (1.41% of the dose per g of brain tissue, not including the content in the vasculature) within 20 min after injection into mice. The amount of anti-transferrin receptor antibody (OX-26), which can be transported into the brain by receptor mediated transcytosis, in the rat brain reached a level of about 0.8% of the injected dose within 1 hr after injection

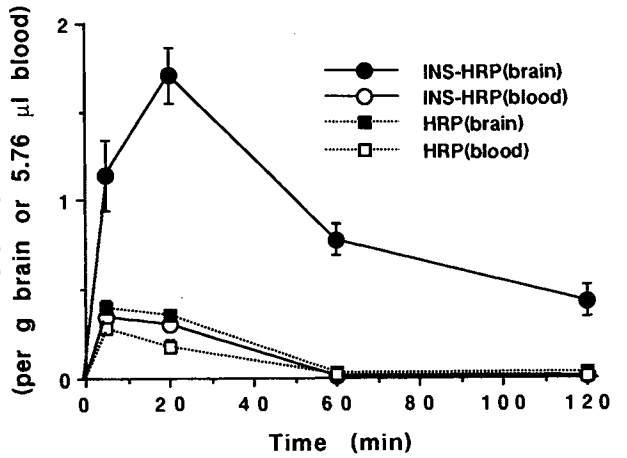

Fig. 1. HRP activity in the brain and blood of mice after i.v. administration of HRP (230 μg) and INS-HRP (213 μg) (mean±SE; n=3). The blood brain volume in mice was 5.76 μl/g calculated from the radioactivity of [^{3}H]-inulin space.

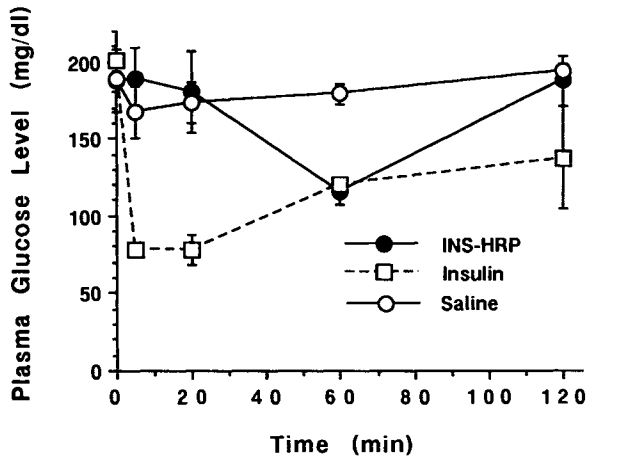

Fig. 2. Plasma glucose levels in mice after i.v. administration of insulin (1.5 μg) and INS-HRP (13 μg, about 1.7 μg as insulin) (mean ±SE; n=3).

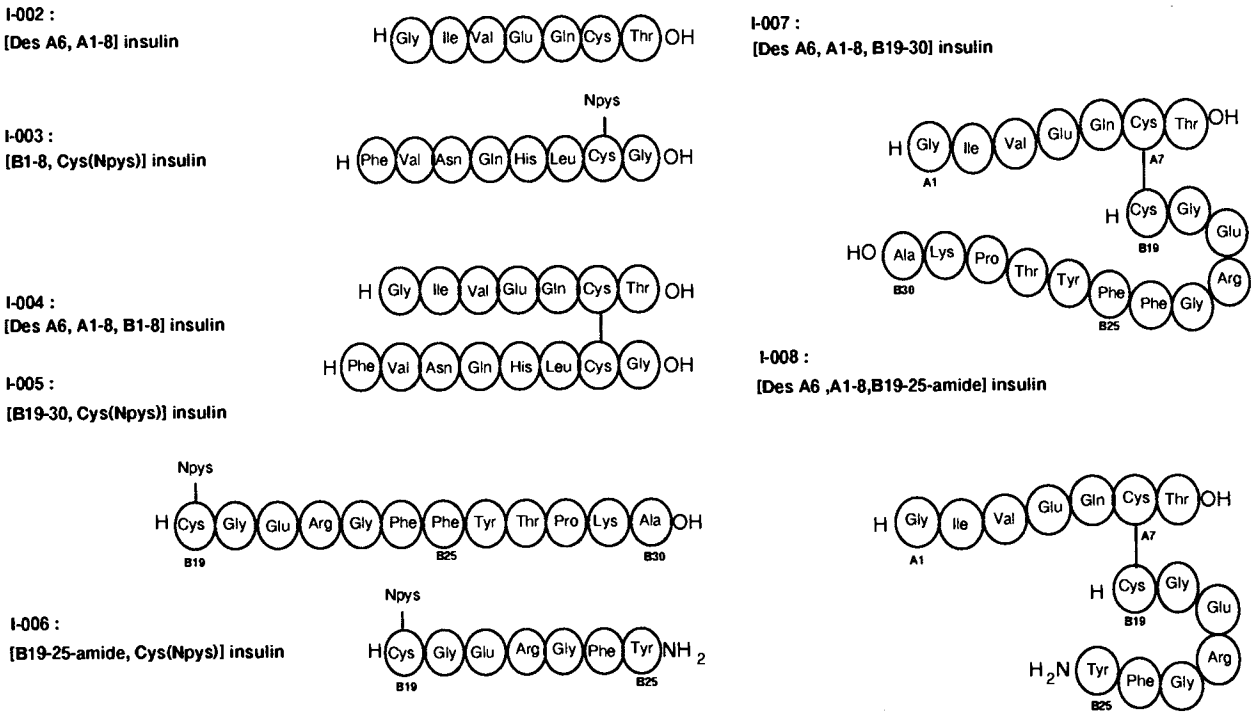

Fig. 3. Amino acid sequences of insulin fragments synthesized chemically.

(12). The amount of uptake in this study therefore seems reasonable with regard to the transport ratio for receptor mediated endocytosis. Although it wasn't determined whether it was HRP or INS-HRP conjugate, a previous study showed that disulfide bonds can be cleaved in the brain (18). Thus, the possibility of brain delivery of peptides and proteins via the insulin receptor using an insulin conjugate prodrug was suggested.

However, insulin has several biological effects on various cells. We measured blood glucose in mice after administration of INS-HRP. As shown in Fig. 2, INS-HRP obviously reduced the glucose levels in mice after i.v. administration, but the levels decreased more slowly than they did after the administration of insulin. This possibly indicates that a period of time is required for cleavage of the conjugate. Since these results confirmed that hypoglycemia would be a possible side effect of using insulin as a carrier for brain delivery, we searched for insulin fragments with good affinity for the receptor but no hypoglycemic activity. Primary cultures of BMEC monolayers (21–23) or isolated brain cap-

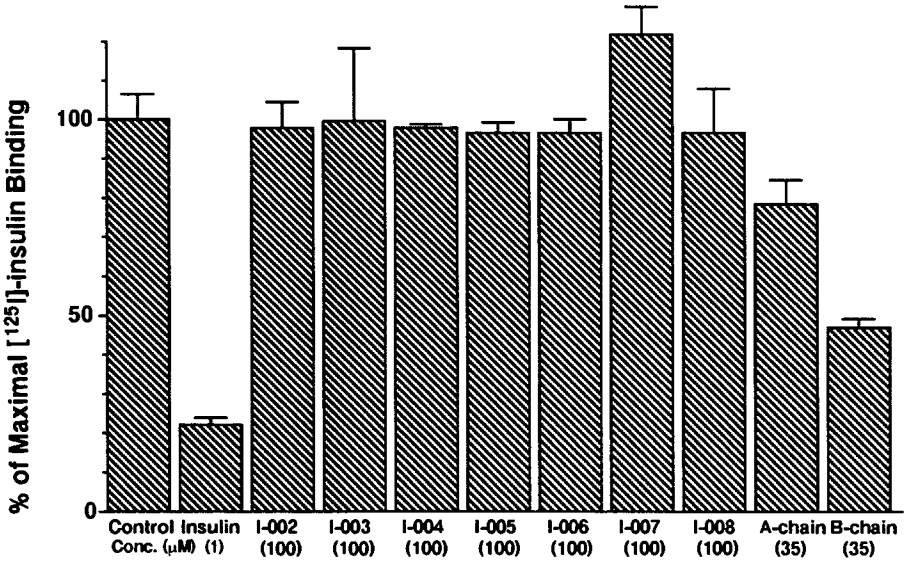

Fig. 4. Competitive effect of the A-chain and B-chain of insulin and various synthesized insulin fragments on the binding of [^{125}I]-insulin (0.025 nM) to BMEC monolayers (mean±SE; n = 3–5).

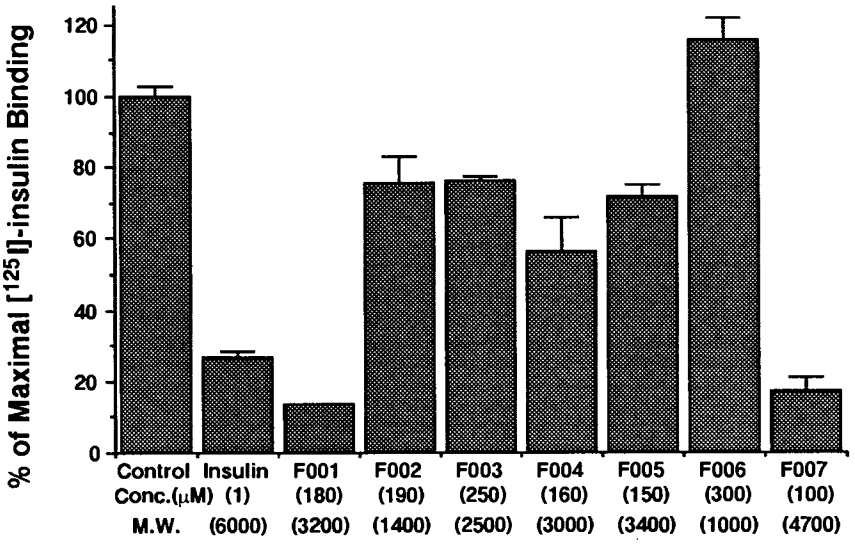

Fig. 5. Competitive effect of various insulin fragments prepared enzymatically on the binding of [^{125}I]-insulin (0.025 nM) to BMEC monolayers. F001, F006 and F007 were prepared using trypsin, and F002, F003, F004 and F005 using V8-protease (mean±SE; n = 3).

illaries (11,24) offer an excellent BBB model. We utilized BMEC to examine the specific binding activity of insulin fragments chemically synthesized and prepared by proteolytic degradation. Given the fact that in four kinds of insulinemia (Phe^{B25} → Leu^{B25}, Chicago (25); Phe^{B24} → Ser^{B24}, Los Angeles (26); Val^{A3} → Leu^{A3}, Wakayama (27) and Tochigi (28)) these congenitally mutant insulins lack binding affinity for the hormone receptor, it is suggested that the C-terminal amino acids of the B chain and the N-terminal amino acids of the A chain play a key role in the binding of insulin to the receptor. First, we determined the binding affinity of the A chain, B chain and chemically synthesized insulin fragments for the insulin receptor on BMEC. The amino acid sequences of the synthesized insulin fragments are shown in Fig. 3: I-002, [DesA6, A1-8] insulin (N-terminal fragment of the A chain); I-003, [B1-8, Cys(Npys)] insulin (N-terminal fragment of the B chain); I-004 [DesA6, A1-8, B1-8] insulin (composite of I-002 and I-003); I-005, [B19-30, Cys(Npys)] insulin (C-terminal fragment of the B chain); I-006, [B19-25-amide, Cys(Npys)] insulin (C-terminal fragment of the B chain); I-007, [DesA6, A1-8, B19-30] insulin (composite of I-002 and I-005); and I-008, [DesA6, A1-8, B19-25-amide] insulin (composite of I-002 and I-006). Fig. 4 shows results of competitive binding experiments using [^{125}I]-insulin and the A chain, B chain and synthesized insulin fragments. Unlike insulin (1 μM), even high concentration of these (A chain and B chain: 35 μM; synthesized insulin fragments: 100 μM) did not inhibit the binding of [^{125}I]-insulin, indicating no binding affinity for the insulin receptor on BMEC. This suggests that the binding regions of insulin need a specific conformational structure for sufficient binding affinity.

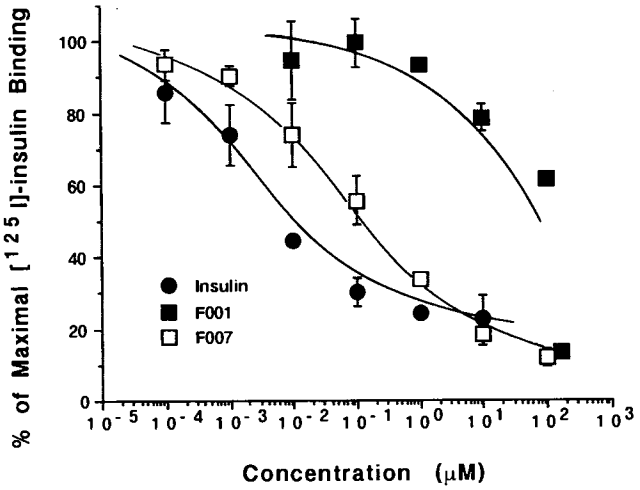

Fig. 6. Competitive effect of F001 and F007 on the binding of [^{125}I]-insulin (0.025 nM) to BMEC monolayers (mean±SE; n = 3).

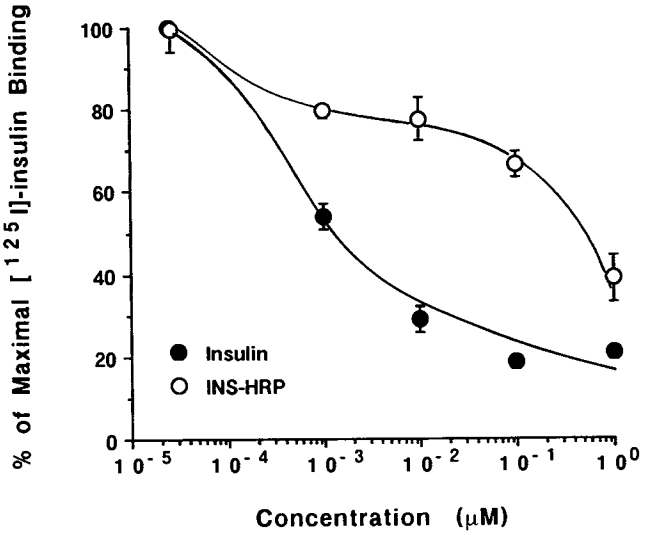

Fig. 7. Competitive effect of INS-HRP on the binding of [^{125}I]-insulin (0.025 nM) to BMEC monolayers (mean±SE; n = 3–5).

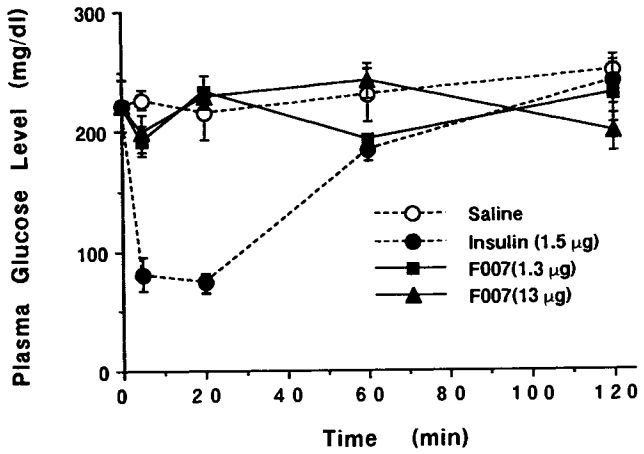

Fig. 8. Plasma glucose levels in mice after i.v. administration of insulin and F007 (mean±SE; n=4).

We next examined the binding activities of insulin fragments prepared by proteolytic degradation (Fig. 5). Unlike insulin (1 μM), even high concentrations of F002–F005 (V8-protease products) and F006 (trypsin product) did not inhibit the binding of [^{125}I]-insulin. On the other hand, F001 and F007 competed significantly with [^{125}I]-insulin for receptor binding. Thereafter, the degree of affinity of F001 and F007 for the insulin receptor on BMEC monolayers was determined at varying concentrations. As shown in Fig. 6, affinity of F001 for the insulin receptor was significantly lower than that of insulin, but that of F007 was much higher than that of F001, but was about one tenth that of insulin. Fig. 7 shows competitive inhibition of the binding of [^{125}I]-insulin to BMEC by INS-HRP conjugate. The affinity of INS-HRP was decreased to less than 1/100 that of insulin, but despite the low affinity for the insulin receptor, HRP activity was obviously detected in the brain after i.v. administration of the conjugate (Fig. 1). These results indicate that F007 has sufficient affinity to be utilized as a carrier to transport non-transportable peptides and proteins across the BBB. Furthermore, insertion of an appropriate spacer between the drug and the carrier or production of a conjugate by a gene engineering technique instead of using a chemical reaction might possibly increase receptor affinity of the conjugate. We measured blood glucose in mice after administration of F007 and insulin. As shown in Fig. 8, F007 did not reduce the glucose level even at 10 times the dose of insulin. These results suggest that F007 will be useful carrier to transport therapeutic peptides across the BBB.

Amino acid sequences of F007 and F001 were determined to be desoctapeptide (B23-30) insulin and des A15-21, des B17-30 insulin, respectively, from the results of amino acid analysis (Fig. 9). The relationship between the structure

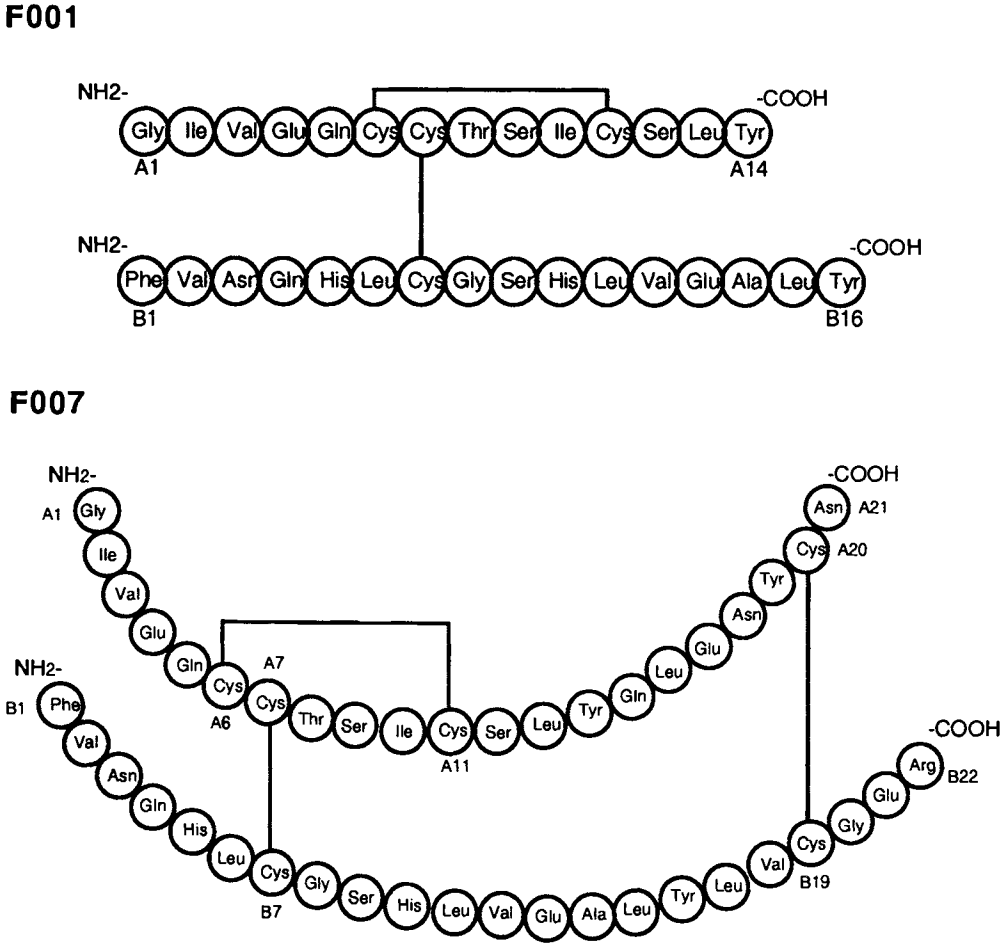

Fig. 9. Assumed amino acid sequences of insulin fragment F001 and F007.

of insulin and its action has long been the subject of investigation. It is known that removal of the C-terminal tetrapeptide, pentapeptide and hexapeptide of the B-chain results in sequential reduction of biological activity (29-31), while desheptapeptide (B24-30) insulin and desoctapeptide (B23-30) insulin are almost completely inactive (32). In our *in vivo* experiment, F007 had no hypoglycemic activity. For desheptapeptide (B24-30) insulinamide and desoctapeptide (B23-30) insulinamide, the relative binding affinity for the receptor in canine hepatocytes is less than 0.2% (33). For insulin fragments lacking the C-terminal peptides or other insulin analogs, relative binding affinity for the receptor in canine hepatocytes correlates with for relative biological activity in isolated rat adipocytes (33). In our experiment, F007, desoctapeptide (B23-30) insulin, had high binding affinity for the receptor on BMEC monolayers, but apparently no affinity for receptors in the hepatocytes which are involved in controlling the blood glucose level. The structural requirements at the C-terminus of the insulin B chain for binding to the receptor on BMEC are possibly different from those for the other insulin receptors. Although we still need to prove transcytosis into the brain (under investigation), the results in the present study suggest that F007 should be useful as a carrier to transport therapeutic peptides and proteins across the BBB.

ACKNOWLEDGMENTS

The authors thank Mr Jeffrey Hogan for valuable comments on the manuscript and Drs. Kenneth L. Audus and Ronald T. Borchardt of the University of Kansas for establishment of the BMEC evaluation system.

REFERENCES

1. J. P. H. Burbach, G. L. Kovacs, D. de Wied, J. W. van Nispen, and H. M. Greven. A major metabolite of arginine vasopressin in the brain is a highly potent neuropeptide. *Science* 221:1310–1312 (1983).
2. S. J. Henriksen, F. E. Bloom, F. McCoy, N. Ling, and R. Guillemin. β-Endorphin induces nonconvulsive limbic seizures. *Proc. Natl. Acad. Sci. USA* 75:5221–5225 (1978).
3. W. Fischer, K. Wictorin, A. Bjorklund, L. R. Williams, S. Varons, and F. H. Grage. Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. *Nature* 329:65–68 (1987).
4. L. R. Williams, S. Varon, G. M. Peterson, K. Wictorin, W. Fischer, A. Bjorklund, and F. H. Gage. Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. *Proc. Natl. Acad. Sci. USA* 83:9231–9235 (1986).
5. J. A. Barranger, S. I. Rapoport, W. R. Fredericks, P. G. Pentchev, K. D. MacDermot, J. K. Steusing, and R. O. Brady. Modification of the blood-brain barrier: Increased concentration and fate of enzymes entering the brain. *Proc. Natl. Acad. Sci. USA* 76:481–485 (1979).
6. R. E. Harbaugh, D. W. Roberts, D. W. Coombs, R. L. Saunders, and T. M. Reeder. Preliminary report: Intracranial cholinergic drug infusion in patients with Alzheimer's disease. *Neurosurgery* 15:514–518 (1984).
7. J. G. Nutt, W. R. Woodward, J. P. Hammerstad, J. H. Carter, and J. L. Anderson. The "on-off" phenomenon in Parkinson's disease: Relation to levodopa absorption and transport. *N. Engl. J. Med.* 310:483–488 (1984).
8. J. E. Jr. Chastain and R. T. Borchardt. L-α-Methyldopa transport across bovine brain microvessel endothelial cell monolayers: A model of the blood-brain barrier. *Neurosci. Res. Commun.* 4:147–152 (1989).
9. R. L. Roberts, R. E. Fine, and A. Sandra. Receptor-mediated endocytosis of transferrin at the blood-brain barrier. *J. Cell. Sci.* 104:521–532 (1993).
10. W. M. Pardridge. Receptor-mediated peptide transport through the blood-brain barrier. *Endocrine Rev.* 7:314–330 (1986).
11. H. J. L. Frank, W. M. Pardridge, W. L. Morris, R. G. Rosenfeld, and T. B. Choi. Binding and internalization of insulin and insulin-like growth factors by isolated brain microvessels. *Diabetes* 35:654–661 (1986).
12. P. M. Friden, L. R. Walus, G. F. Musso, M. A. Taylor, B. Malfroy, and R. M. Starzyk. Anti-transferrin receptor antibody and antibody-drug conjugates cross the blood-brain barrier. *Proc. Natl. Acad. Sci. USA* 88:4771–4775 (1991).
13. P. M. Friden, L. R. Walus, P. Watson, S. R. Doctrow, J. W. Kozarich, C. Bäckman, H. Bergman, B. Hoffer, F. Bloom, and A.-C. Granholm. Blood-brain barrier penetration and *in vivo* activity of an NGF Conjugate. *Science* 259:373–377 (1993).
14. A.-C. Granholm, C. Bäckman, F. Bloom, T. Ebendal, G. A. Gerhardt, B. Hoffer, L. Mackerlova, L. Olson, S. Söderström, L. R. Walus, and P. M. Friden. NGF and anti-transferrin receptor antibody conjugate: Short and long-term effects on survival of cholinergic neurons in intraocular septal transplants. *J. Pharmacol. and Exp. Ther.* 268:448–459 (1994).
15. U. Bickel, T. Yoshikawa, E. M. Landaw, K. F. Faull, and W. M. Pardridge. Pharmacologic effects *in vivo* in brain by vector-mediated peptide drug delivery. *Proc. Natl. Acad. Sci. USA* 90:2618–2622 (1993).
16. E. E. Schneeberger, Circulating proteins and macromolecular transport across continuous nonfenestrated endothelium. *Ann. N. Y. Acad. Sci.* 401:25–37 (1982).
17. K. R. Smith and R. T. Borchardt. Permeability and mechanism of albumin, cationized albumin and glycosylated albumin transcellular transport across monolayers of cultured bovine brain capillary endothelial cells. *Pharm. Res.* 6:466–473 (1989).
18. W. M. Pardridge, D. Triguero, and J. L. Buciak. β-Endorphin chimeric peptides: Transport through the blood-brain barrier *in vivo* and cleavage of disulfide linkage by brain. *Endocrinology* 126:977–984 (1990).
19. D. Triguero, J. L. Buciak, J. Yang, and W. M. Pardridge. Blood-brain barrier transport of cationized immunoglobulin G: Enhanced delivery compared to native protein. *Proc. Natl. Acad. Sci. USA* 86:4761–4765 (1989).
20. Y. Kiso, Y. Fujiwara, T. Kimura, and M. Yoshida. A new method for solid phase peptide synthesis by α-amino deprotecting procedure using methanesulfonic acid. *Peptide Chemistry* 1988:123–128 (1989).
21. K. L. Audus and R. T. Borchardt. Characterization of an *in vitro* blood-brain barrier model system for studying drug transport and metabolism. *Pharm. Res.* 3:81–87 (1986).
22. K. L. Audus and R. T. Borchardt. Bovine brain microvessel endothelial cell monolayers as a model system for the blood-brain barrier. *Ann. N. Y. Acad. Sci.* 507:9–18 (1987).
23. K. L. Audus, R. L. Bartel, I. J. Hidalgo, and R. T. Borchardt. The use of cultured epithelial and endothelial cells for drug transport and metabolism studies. *Pharm. Res.* 7:435–451 (1990).
24. K. R. Duffy, W. M. Pardridge, and R. G. Rosenfeld. Human blood-brain barrier insulin-like growth factor receptor. *Metabolism* 37:136–140 (1988).
25. S. C. M. Kwok, D. F. Steiner, A. H. Rubenstein, and H. S. Tager. Identification of a point mutation in the human insulin gene giving rise to a structurally abnormal insulin (Insulin Chicago). *Diabetes* 32:872–875 (1983).
26. M. Haneda, S. J. Chan, S. C. M. Kok, A. H. Rubenstein, and D. F. Steiner. Studies on mutant human insulin gene: Identification and sequence analysis of a gene encoding [Ser^{B24}] insulin. *Proc. Natl. Acad. Sci. USA* 80:6366–6370 (1983).
27. K. Nanjo, T. Sanke, M. Miyano, K. Okai, R. Sowa, M. Kondo, S. Nishimura, K. Iwo, K. Miyamura, B. D. Given, S. J. Chan, H. S. Tager, D. F. Steiner, and A. H. Rubenstein. Diabetes due to secretion of a structurally abnormal insulin (insulin

Wakayama): Clinical and functional characteristics of [Leu^{A3}]-insulin. *J. Clin. Invest.* 77:514–519 (1986).
28. Y. Iwamoto, H. Sakura, Y. Ishii, R. Yamamoto, S. Kumakura, Y. Sakamoto, A. Matsuda and T. Kuzuya. A new case of abnormal insulinemia with diabetes: Reduced insulin values determined by radioreceptor assay. *Diabetes* 35:1237–1242 (1986).
29. F. M. Ng, S. Q. Zhu, D. F. Cui, L. Fan, Y. D. Huang, and Y. S. Zhang. Structure and activity of B-chain of insulin. *Biochem. Int.* 18:373–381 (1989).
30. C. S. Cockram, R. H. Jones, P. H. Sonksen, M. A. Tatnell, S. Q. Zhu, and G. Dodson. An examination of the role of insulin dimerisation and negative cooperativity using the biological properties of the despentapeptide and deshexapeptide insulins. *Diabetologia* 30:733–738 (1987).
31. P. G. Katsoyanis, J. Ginos, A. Cosmatos, and G. Schwartz. Synthesis of destetrapeptide B$^{27-30}$ human (porcine) insulins. A biological active insulin analog. *J. Am. Chem. Soc.* 95:6427–6434 (1973).
32. S. Q. Zhu. Studies on structure and biological activity of insulin. In R. A. Bradshow and J. Tang (eds.), *Molecular Architecture of Proteins and Enzymes*, Academic Press, New York, 1985, pp. 185–197.
33. S. H. Nakagawa and H. S. Tager. Role of the phenylalanine B25 side chain in directing insulin interaction with its receptor. *J. Biol. Chem.* 261:7332–7341 (1986).