Vol. 146, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1987 Pages 307-313

CHIMERIC PEPTIDES AS A VEHICLE FOR PEPTIDE PHARMACEUTICAL DELIVERY THROUGH THE BLOOD-BRAIN BARRIER

William M. Pardridge, Arno K. Kumagai, and Jody B. Eisenberg

Department of Medicine,
Division of Endocrinology,
UCLA School of Medicine,
Los Angeles, California 90024

Received March 19, 1987

A new strategy for peptide delivery through the brain capillary wall, i.e., the blood-brain barrier (BBB), is the synthesis of chimeric peptides which are formed by the covalent coupling of a non-transportable peptide (e.g., β-endorphin) to a transportable peptide that undergoes receptor- or absorptive-mediated transcytosis at the BBB. β-endorphin was covalently coupled via disulfide linkage to cationized albumin (pI ≥ 9) which, owing to it's highly basic charge, undergoes rapid absorptive-mediated transport into brain from blood. The [^{3}H]labeled β-endorphin-cationized albumin chimera was rapidly taken up by isolated brain capillaries in vitro and by rat brain in vivo; conversely, the BBB uptake of native [^{3}H]β-endorphin was negligible. The synthesis of chimeric peptides is a new strategy for solving the problem of peptide delivery through the BBB.

Strategies for peptide pharmaceutical delivery through the brain capillary wall, i.e., the blood-brain barrier (BBB), that have been tried in the past are largely invasive and are probably not practical for use in large numbers of patients, e.g., opening of the BBB via carotid injections of hypertonic solutions or placement of an intraventricular catheter through the skull (1, 2). Therefore, a limiting factor in the development of peptides as neuropharmaceuticals is the problem of drug delivery through the BBB. Strategies for pharmaceutical delivery through the BBB that are most successful are those that rely on normal brain capillary transport processes (3). For example, brain dopamine can be elevated in Parkinson's disease by the administration of it's precursor amino acid, L-dopa (4), which undergoes carrier-mediated transport through the BBB (5). With regard to peptides, recent studies indicate that both receptor-mediated and

0006-291X/87 $1.50

absorptive-mediated transcytosis of peptides through the BBB occurs (6). Peptides in the circulation, such as insulin, insulin-like growth factor (IGF)-II, or transferrin, are believed to undergo receptor-mediated transcytosis through the BBB (6, 7). Substances such as cationized albumin undergo absorptive-mediated transcytosis through the BBB on the basis of electrostatic interactions between the positively charged protein and negative charges lining the glycocalyx of the brain capillary endothelium (8). Conversion of native albumin (pI = 4) to cationized albumin (pI = 9) also results in rapid entry of the protein into cerebrospinal fluid (9), presumably via absorptive-mediated transcytosis through the blood-CSF barrier, i.e., the choroid plexus.

On the basis of this new information regarding mechanisms for peptide transport through the BBB, it has been postulated that the preparation of chimeric peptides provides a new strategy for peptide delivery through the BBB (3, 6). Chimeric peptides are formed when a transportable peptide (e.g., insulin, IGF-II, transferrin, or cationized albumin) is covalently coupled to a nontransportable peptide (e.g., β-endorphin, an interferon, an interleukin, or an antibody fragment) often via a bond that is cleavable by the brain in vivo, (e.g., disulfide bonds). The present studies investigate whether coupling of a nontransportable peptide, e.g., β-endorphin, to a transportable peptide, e.g., cationized albumin, would allow for uptake into brain capillaries of the chimeric peptide. Cationized albumin was chosen as the model for nontransportable peptides because, unlike insulin, transferrin, or IGF-II, this peptide may not have any major endogenous biological function.

METHODS

Materials - Bovine albumin (Pentex Fraction V) was obtained from Miles Laboratories (Elkhart, IN). Human β-endorphin was obtained from Peninsula Laboratories (Belmont, CA). N-succinimidyl 3-(2-pyridyldithio)proprionate (SPDP) was purchased from Pierce Chemical Company (Rockford, IL). [^{3}H]$NaBH_4$ and [^{14}C]sucrose were obtained from New England Nuclear Corporation (Boston, MA). Polybuffer exchanger 94 resin, polybuffer 96 elution buffer, and Sepharose CL6B were purchased from Pharmacia (Piscataway, NJ). All other reagents were obtained from Sigma Chemical Company (St. Louis, MO).

Chimeric synthesis - Cationized albumin was prepared by covalent coupling of hexamethylenediamine (HMD) to bovine albumin (10). Ten ml of a 10% solution of albumin in water was slowly added to 60 ml of 2 M HMD and the pH of the solution was adjusted to 6.5 with 1 N HCl. After 30 minutes, 1 g of N-ethyl-N'-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDAC) was added to activate the carboxyl groups of the albumin followed by the addition

of another 1 g EDAC 1 hour later. The pH was adjusted to 6.5 with 0.2 N HCl. The solution was allowed to mix overnight with constant stirring. The next day the solution was dialyzed extensively against distilled water and the material was then purified by chromatofocussing using the Pharmacia polybuffer exchanger 94 resin and the polybuffer 96 elution buffer (11). The major protein peak eluted in the void volume of the column, indicating the pI of the cationized albumin was ≥ 9. The human β-endorphin was tritiated by reductive methylation of lysine residues to a specific activity of 3 μCi/μg using [^{3}H]sodium borohydride ($NaBH_4$) exactly as described previously for native bovine albumin (12). Unreacted [^{3}H]$NaBH_4$ was removed by dialysis, and the trichloroacetic acid precipitability of the labeled endorphin was 98%. The [^{3}H]endorphin was coupled to lysine residues on the cationized albumin using the bifunctional cleavable cross linking reagent, SPDP (13). Five mg of chromatofocussed cationized albumin was dissolved in 0.1 ml of 0.1 M PBS (0.1 M Na_2HPO_4, 0.15 M NaCl, pH = 7.5) followed by the addition of 10 μL 20 mM SPDP dissolved in 99.5% ethanol. Following incubation at room temperature for 30 minutes, the solution was acidified to pH = 4.5 with 0.5 M Na acetate followed by the addition of 1/10 volume of 0.25 M dithiothreitol. Following a 30 minute incubation at room temperature, the solution underwent three consecutive 30 minute dialyses against 2 L of 0.1 M PBS. Separately, [^{3}H]β-endorphin was resuspended in 0.2 ml of 0.1 N HCl containing 0.2 mg β-endorphin. The pH was adjusted to 7 with 1 N NaOH and 10 μL of 20 mM SPDP was added. After a 30 minute incubation at room temperature, the solution underwent three sequential 15 minute dialyses against 0.1 M PBS using Visking tubing with a 1,000 molecular weight pore size. The final cationized albumin and β-endorphin dialysands were combined and shaken overnight at room temperature. The [^{3}H]β-endorphin covalently coupled to the cationized albumin was separated from [^{3}H]β-endorphin nonspecifically adsorbed to the cationized albumin by elution through a 1.6 x 80 cm Sepharose CL6B column in 0.1 M PBW (Na_2HPO_4, pH = 8.0) containing 6 M guanidine. The column was eluted at a rate of 9 ml/hour and 2 ml fractions were collected. The fractions were then counted for [^{3}H] and absorbance at 280 nm was measured. The conjugated β-endorphin eluted at fraction 21, whereas the unreacted β-endorphin eluted at fraction 62. Incubation of the conjugate with 25 mM dithiothreitol (pH = 4.5) for 30 minutes at room temperature resulted in the complete cleavage of the disulfide linkage and migration of all radioactivity at fraction 62 (Figure 1, inset).

Uptake of chimeric peptide by isolated brain capillaries - Capillaries were isolated from fresh bovine brain using a mechanical homogenization technique as described previously (14). This method results in the preparation of pure brain capillaries that are free of adjoining brain tissue. The uptake of free [^{3}H]β-endorphin, and [^{3}H]β-endorphin conjugated to cationized albumin with SPDP by the isolated bovine brain capillaries was determined by incubating 25 - 100 μg_p of capillaries with 0.03 μCi/ml of free [^{3}H]β-endorphin or [^{3}H]conjugated β-endorphin in 0.45 ml of Ringer's-Hepes buffer (RHB, 10 mM Hepes, pH = 7.4) containing 0.1% native bovine albumin for 5 seconds to 30 minutes at room temperature. After the incubation, 400 μL of the incubation solution was transferred to a small Beckman microfuge tube and was microfuged for 45 seconds at room temperature. The supernatant was aspirated, the tip containing the capillary pellet was solubilized in 0.5 ml 1 N NaOH, and the solution was counted for [^{3}H] by routine liquid scintillation spectrometry and the protein content was determined by the method of Lowry et al (15). Metabolism of the chimeric peptide was assessed by pooling three supernatants following a 10 minute incubation at room temperature and adding guanidine to a final concentration of 6 M. The solution was stored overnight at 4°C and was then applied to the Sepharose CL6B column as described

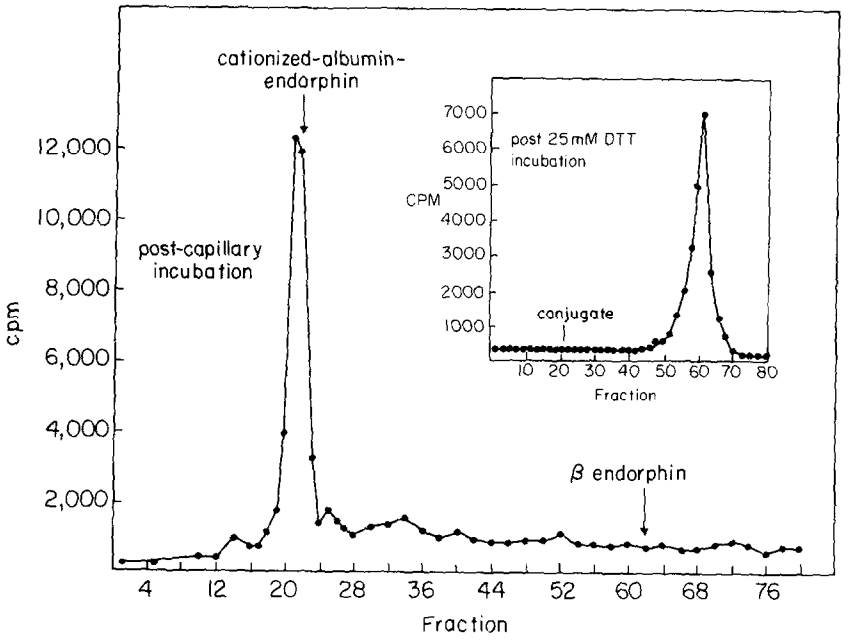

Figure 1. Migration of [^{3}H]β-endorphin-SPDP-cationized albumin chimeric peptide through a 1.6 x 78 cm Sepharose CL6B column [elution with 6 M guanidine and 0.1 M phosphate buffer (pH = 8.0)] after a 10 minute incubation of the chimera with isolated bovine brain capillaries at 23°C. Virtually all [^{3}H] radioactivity eluted at fraction 21, which is where [^{3}H]cationized albumin elutes. Unreacted β-endorphin migrated at fraction 62. [Fractions = 2.2 ml] (Inset) The [^{3}H]β-endorphin-SPDP-cationized albumin chimeric peptide was incubated in 25 mM dithiothreitol (DTT) in 25 mM sodium acetate buffer (pH = 4.5) for 30 minutes at room temperature. The material was then applied to the CL6B column and eluted in the 6 M guanidine phosphate buffer, and all of the radioactivity migrated at fraction 62, consistent with complete thiol-based cleavage of the chimeric peptide by the dithiothreitol.

above. Internalization of [^{3}H]β-endorphin coupled to cationized albumin was determined with a mild acid wash technique as described previously (14).

In vivo brain uptake - 0.15 ml solutions of RHB containing [^{3}H]β-endorphin (either free or conjugated to cationized albumin), 1.5 μCi/ml, [^{14}C]sucrose, 0.5 μCi/ml, and 0.1 g/dl native bovine albumin were rapidly injected into the carotid artery of ketamine-anesthetized adult Sprague-Dawley rats (16). After 15 seconds, i.e., a time sufficient for a single pass of the bolus through the brain, the rats were decapitated and the hemisphere ipsilateral to the injection was removed and solubilized in Soluene 350 (Packard Instrument Company) for [^{3}H], [^{14}C] double isotope liquid scintillation spectrometry. The brain uptake index (BUI) was computed from the ratio of [^{3}H]/[^{14}C] in the brain divided by the same ratio in the injection solution.

RESULTS AND DISCUSSION

The uptake of free [^{3}H]β-endorphin and the β-endorphin conjugated to cationized albumin with SPDP by the isolated bovine brain capillaries in vitro is shown in Figure 2. There was no measurable uptake

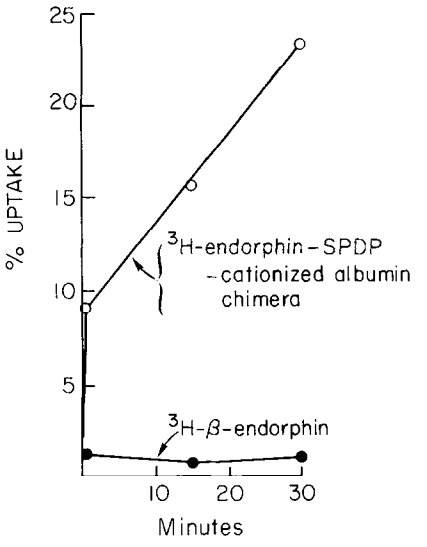

Figure 2. The precent [^{3}H]endorphin-SPDP-cationized albumin chimera or native [^{3}H]β-endorphin uptake by the brain capillaries is plotted versus incubation time. The individual incubation flasks contained approximately 40 µg of capillary protein. The [^{3}H]chimeric peptide taken up by the brain capillaries represents both membrane-bound and endocytosed peptide since more than 35% of the radioactivity associated with the capillaries was resistant to a mild acid wash [25 mM sodium acetate, pH = 3.5, in 0.12 M saline, 6 minutes at 4°C (14)].

of the β-endorphin, consistent with it's known lack of transport through the BBB (17). Conversely, the uptake of the endorphin-conjugated cationized albumin was rapid and increased with incubation time. The uptake of the endorphin conjugated to cationized albumin increased with the amount of capillary protein in the reaction vessel (Figure 3), and this uptake was not inhibited by 25 mg/ml bovine serum albumin (BSA) or 10 µg/ml of human β-endorphin, but was markedly inhibited by 100 µg/ml of cationized bovine serum albumin (cBSA) as shown in Figure 3. The stability of the chimeric disulfide bond during a 10 minute incubation with the bovine brain capillaries at room temperature was assessed by preparing a 6 M guanidine extract of the microvessel pellet (Methods), and the supernatant of this extract was applied to the CL6B column and eluted with 6 M guanidine in 0.1 M PBW. As shown in Figure 1, the [^{3}H]β-endorphin conjugated to the cationized albumin, eluted at fraction 21, indicating no measurable breakdown of the chimeric peptide during the capillary incubation.

The in vivo uptake of the [^{3}H]β-endorphin coupled to the cationized albumin was rapid compared to the in vivo brain uptake of free [^{3}H]β-endorphin. The first pass brain uptake of the [^{3}H]endorphin chimera, relative to [^{14}C]sucrose, after a single

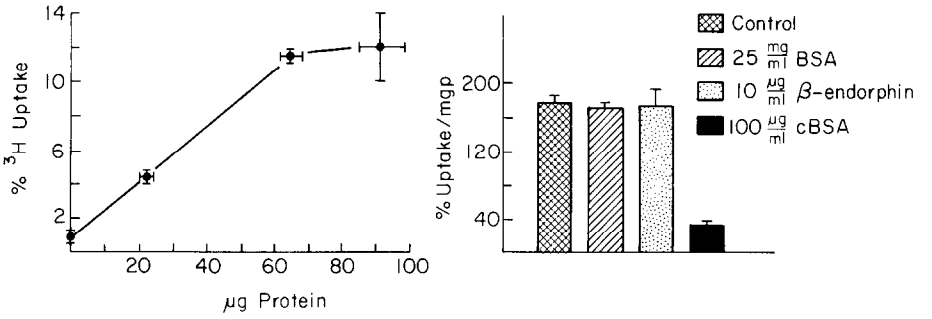

Figure 3. (Left) The percent uptake of [^{3}H]β-endorphin-SPDP-cationized albumin chimeric peptide is plotted versus the μg of bovine brain capillary protein in the individual incubation tubes. The percent uptake of the chimera increased in proportion to the amount of brain capillary. (Right) The percent uptake (per mg protein of bovine brain capillary) of [^{3}H]β-endorphin-SPDP-cationized albumin chimera is measured at 4°C for 10 minute incubations in the presence of buffer alone (control), 25 mg/ml bovine serum albumin (BSA), 10 μg/ml β-endorphin, or 100 μg/ml cationized bovine albumin (cBSA). Only the cationized albumin inhibited the uptake of the chimera, whereas native albumin and unconjugated β-endorphin had no effect.

carotid injection of the two isotopes was 12.7 ± 1.9 (mean ± S.E., n = 4 rats), indicating the uptake by rat brain of the β-endorphin coupled to cationized albumin was nearly thirteen-fold greater than the uptake of the vascular marker, [^{14}C]sucrose. In contrast, the first pass brain uptake of native [^{3}H]β-endorphin, relative to [^{14}C]sucrose, was only 1.8 ± 0.1 (mean ± S.E., n = 3 rats), consistent with the known poor penetration of the native β-endorphin through the BBB.

In summary, these studies provide the basis for a new paradigm for physiologic based peptide delivery through the BBB that is potentially applicable to a wide variety of pharmaceuticals in neurologic diseases. The use of disulfide based bonds to form the chimeric peptide conjugate is advantageous, since this bond is relatively stable in plasma but is cleaved in tissues by disulfide reductases (18). The latter enzymes are apparently not abundant in brain capillary endothelia, since the chimera was relatively stable after incubation with bovine brain capillaries (Figure 1). The use of physiologic-based strategies for circumventing the transport barrier at the brain capillary and the administration of peptides via intranasal insufflation (to circumvent the gastrointestinal barrier) (19) provides a rational approach to solving the peptide delivery problem and for the development of peptides as promising neuropharmaceuticals (3, 6).

ACKNOWLEDGEMENT

The authors wish to thank Dawn Brown for skillfull preparation of the manuscript, and Dr. Tetsuya Terasaki for technical assistance.

REFERENCES

1. Barranger, J.A., Rapoport, S.I., Fredericks, W.R., Pentchev, P.G. MacDermot, K.D., Steusing, J.K., and Brady, R.O. (1979) Proc. Natl. Acad. Sci., U.S.A. 76:481-485.
2. Harbaugh, R.E., Roberts, D.W., Coombs, D.W., Saunders, R.L., and Reeder, T.M. (1984) Neurosurgery 15:514-518.
3. Pardridge, W.M. (1985) In Directed Drug Delivery: A Multi-disciplinary Problem (R.P. Borchardt, A.J. Repta, and V.J. Stella, eds.), pp. 83-96. Humana Press, Inc., Clifton, New Jersey.
4. Mena, I. and Cotzias, G.C. (1975) N. Engl. J. Med. 292:181-184.
5. Oldendorf, W.H. (1971) Am. J. Physiol. 221(6):1629-1639.
6. Pardridge, W.M. (1986) Endocrine Rev. 7:314-330.
7. Duffy, K.R. and Pardridge, W.M. (1987) Brain Res., in press.
8. Schneeberger, E.E. (1982) Ann. N.Y. Acad. Sci. 401:25-37.
9. Griffin, D.E. and Giffels, J. (1982) J. Clin. Invest. 70:289-295.
10. Bergmann, P., Kacenelenbogen, R., and Vizet, A. (1984) Clinical Science 67:35-43.
11. Keenan, M.J. and Holmes, R.P. (1985) Biochem. J. 229:669-674.
12. Pardridge, W.M., Eisenberg, J., and Cefalu, W.T. (1985) Am. J. Physiol. 249:E264-E267.
13. Carlsson, J., Drevin, H., and Axen, R. (1978) Biochem. J. 173: 723-737.
14. Pardridge, W.M., Eisenberg, J., and Yamada, T. (1985) J. Neurochem. 44:1178-1184.
15. Lowry, O.H., Rosebrough, H.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193:262-275.
16. Pardridge, W.M. and Fierer, G. (1985) J. Cereb. Blood Flow Metab. 5:275-281.
17. Houghten, R.A., Swann, R.W., and Li, C.H. (1980) Proc. Natl. Acad. Sci. U.S.A. 77:4588-4591.
18. Letvin, N.L., Goldmacher, V.S., Ritz, J., Yetz, J.M., Schlossman, S.F., and Lambert J.M. (1986) J. Clin. Invest. 77:977-984.
19. Salzman, R., Manson, J.E., Griffing, G.T., Kimmerle, R., Ruderman, N., McCall, A., Stoltz, E.I., Mullin, C., Small, D., Armstrong, J., and Melby, J.C. (1985) N. Engl. J. Med. 312:1078-1084.