# Absorptive-Mediated Endocytosis of an Adrenocorticotropic Hormone (ACTH) Analogue, Ebiratide, into the Blood–Brain Barrier: Studies with Monolayers of Primary Cultured Bovine Brain Capillary Endothelial Cells

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The internalization of a neuromodulatory adrenocorticotropic hormone (ACTH) analogue, [$^{125}$I]ebiratide (H-Met($O_2$)-Glu[$^{125}$I]His-Phe-D-Lys-Phe-NH($CH_2$)$_8$$NH_2$), was examined in cultured monolayers of bovine brain capillary endothelial cells (BCEC). HPLC analysis of the incubation solution showed that [$^{125}$I]ebiratide was not metabolized during the incubation with BCEC. The acid-resistant binding of [$^{125}$I]ebiratide to BCEC increased with time for 120 min and showed a significant dependence on temperature and medium osmolarity. Pretreatment of BCEC with dansylcadaverine or phenylarsine oxide, endocytosis inhibitors, and 2,4-dinitrophenol, a metabolic inhibitor, decreased significantly the acid-resistant binding of [$^{125}$I]ebiratide. The acid-resistant binding of [$^{125}$I]ebiratide was saturable in the presence of unlabeled ebiratide (100 n*M*–1 m*M*). The maximal internalization capacity ($B_{max}$) at 30 min was 7.96 ± 3.27 pmol/mg of protein with a half-saturation constant ($K_d$) of 15.9 ± 6.4 μ*M*. The acid-resistant binding was inhibited by basic peptides such as poly-L-lysine, protamine, histone, and ACTH but was not inhibited by poly-L-glutamic acid, insulin, or transferrin. These results confirmed that ebiratide is transported through the blood–brain barrier via an absorptive-mediated endocytosis.

KEY WORDS: ebiratide; adrenocorticotropic hormone analogue; adsorptive endocytosis; absorptive-mediated endocytosis; blood–brain barrier (BBB) transport; internalization; primary cultured brain capillary endothelial cells; peptide.

## INTRODUCTION

Peptide drugs acting on the central nervous system should possess a high affinity for receptors in the brain, efficient transport through the blood–brain barrier (BBB), and stability against peptidases. In previous studies on a dynorphin-like analgesic peptide, E-2078 (1,2), chemical modifications of the native dynorphin were successful in overcoming these difficulties. Absorptive-mediated endocytosis (AME or adsorptive endocytosis) at the capillary endothelial cells was shown to be a useful pathway for the transport of E-2078, a basic peptide, through the BBB (1).

A candidate drug for treatment of an Alzheimer type of dementia is ebiratide [H-Met($O_2$)-Glu-His-Phe-D-Lys-Phe-NH($CH_2$)$_8$$NH_2$], designed by chemical modification of an adrenocorticotropic hormone (ACTH) (3–5). Though the primary structure of ebiratide is different from that of E-2078 (1), both peptides have the same isoelectric point, i.e., 10 (1,6), a parameter known to affect peptide AME at the BBB (1,6–9). Based on the results obtained from whole animals (10) and *in vitro* with isolated brain capillaries (6), we have postulated previously that ebiratide crosses the BBB via AME after systemic administration.

Recently, the utility of primary cultured brain capillary endothelial cells (BCEC) was demonstrated for studies on carrier-mediated transport of drugs (11,12) and nutrients (11,13,14). The viability of BCEC is known to be high, while that of isolated brain capillaries is not. Therefore, to confirm that ebiratide is transported through the BBB via AME, we have performed a transport study using bovine BCEC, because endocytosis and exocytosis systems are known to depend on an adequate metabolic capacity (15–17).

## MATERIALS AND METHODS

### Chemicals

Ebiratide [H-Met($O_2$)-Glu-His-Phe-D-Lys-Phe-NH($CH_2$)$_8$$NH_2$] synthesized at Hoechst AG (Frankfurt, Germany) was used for this study. [$^{14}$C(U)]Sucrose (185 MBq/mmol) and Na$^{125}$I were purchased from New England Nuclear (Boston, MA). Horse serum was purchased from Hazleton Biologics Inc. (St. Lenexa, USA) or GIBCO (Grand Island, NY). Rat tail collagen (type I) was purchased from Collaborative Research Inc. (Bedford, MA), and human fibronectin from Boehringer Mannheim GmbH (Mannheim, Germany). Salmon roe protamine sulfate was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and human holotransferrin from Green Cross Corp. (Osaka, Japan). Porcine insulin, poly-L-lysine hydrobromide, poly-L-glutamic acid sodium salt, human ACTH, calf thymus histone VS (lysine rich), bovine serum albumin (Fraction V), and phenylarsine oxide were purchased from Sigma Chemical Co. (St. Louis, MO), and dansylcadaverine, from Seikagaku Kogyo Co., Ltd. (Tokyo). All other chemicals were of reagent grade and commercially available.

### Radioiodination of Ebiratide

Ebiratide was labeled with $^{125}$I by the chloramine-T method (18), as follows. Ebiratide, 10 μl (1 mg/ml), was mixed with 18.5 MBq of Na$^{125}$I and 50 μl of 0.5 *M* phosphate buffer (pH 7.4) in a polyethylene tube, and 10 μl of 0.25% chloramine-T was added and allowed to react at room temperature for 30 sec. The reaction was stopped by the addition of 0.25% sodium metabisulfite (25 μl), and 10 μl of 10% KI was added as a carrier. The reaction mixture was purified by high-performance liquid chromatography (HPLC). The

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HPLC conditions were as follows: column, VYDAC 218TP5415 (The Separations Group Co., Ltd., Hasperia, USA); mobile phase, a mixture of water, acetonitrile, and trifluoroacetate (80:15:0.1); and flow rate, 1.0 ml/min. For detection, a radiodetector (Model-170, Beckman Co., Ltd., Palo Alto, CA) was used. [$^{125}$I]Ebiratide obtained has a specific activity of about 37 TBq/g and a chemical purity of >95%.

### Isolation and Culture of BCEC

Brain capillary endothelial cells were isolated from cerebral gray matter of bovine brains as described by Audus and Borchardt (19) with minor modifications. Details of the preparation and culture have been described in the previous report (11). The isolated BCEC were stored at −100°C in culture medium containing 20% horse serum and 10% dimethyl sulfoxide (DMSO) until use for cell culture. Prior to seeding, four-well dishes were coated with rat tail collagen under UV light and then with human fibronectin. Isolated BCEC were seeded on the dishes and cultured at 37°C with 95% air and 5% $CO_2$. Transport experiments were performed when cells reached confluence in 10–12 days. These cultured cells were identified to be capillary endothelial cells by the immunostaining method using Factor VIII related antigen (20) (data not shown).

### HPLC Analysis

Unchanged [$^{125}$I]ebiratide and its metabolites were separated by HPLC from the incubation solution and from the acid-resistant fraction after the incubation of [$^{125}$I]ebiratide with BCEC. The incubation solution was applied to the HPLC system without further treatment. The fraction of acid-resistant binding was treated with 1 *N* NaOH to solubilize cells. Each sample was, when necessary, evaporated to dryness by centrifugal evaporation under reduced pressure and reconstituted with the mobile phase of the assay. Then 50 μl of this solution was applied to the HPLC system. The HPLC conditions were as follows: column, VYDAC 214TP54; mobile phase, 1% phosphoric acid adjusted to pH 3.0 with triethylamine and acetonitrile (8:1); and flow rate, 1.0 ml/min. The eluents were collected by the fraction collector FRAC-100 (Pharmacia, Tokyo) and the radioactivity in each eluent (0.5 ml) was counted using a γ-counter, ARC-600 (Aloka Co., Ltd., Tokyo).

### Internalization Studies Using Cultured BCEC

Uptake of [$^{125}$I]ebiratide into cultured monolayers of BCEC was examined by a method reported previously (21) with minor modifications. Briefly, cultured cell monolayers were washed three times with 1 ml of incubation solution (141 m*M* NaCl, 4 m*M* KCl, 2.8 m*M* $CaCl_2$, 1 m*M* $MgSO_4$, 10 m*M* D-glucose, 10 m*M* HEPES, 0.1% bovine serum albumin, pH 7.4, adjusted with 1 *N* NaOH, 300 mOsm) at 37°C. The uptake experiment was initiated by adding 250 μl of incubation solution containing [$^{125}$I]ebiratide (5.0 μCi) or [$^{14}$C]sucrose (4.0 μCi) to cells in the absence or presence of various compounds. [$^{14}$C]Sucrose was used as the extracellular space marker. At designated times after incubation, cells were washed three times with 1 ml of the ice-cold incubation solution, and uptake was stopped.

An acid wash technique (1,6–9) was then used to estimate the internalized amount of [$^{125}$I]ebiratide into BCEC. The acid treatment removes [$^{125}$I]ebiratide bound to the cell surface. After the above uptake procedure, cells were incubated for 10 min with 1 ml of ice-cold acetate–barbital buffer (28 m*M* $CH_3COONa$, 120 m*M* NaCl, 20 m*M* barbital, pH 3.0, adjusted with 1 *N* HCl, 320 mOsm) at 4°C. Then the buffer was removed, and cells were subsequently washed four more times with 1 ml of acetate–barbital buffer. The radioactivity in the cells was measured after solubilization by 250 μl of 1 *N* NaOH for 1 hr at room temperature and represents internalized [$^{125}$I]ebiratide. Radioactivity was counted by a γ-counter for $^{125}$I and a liquid scintillation counter, LSC-700 (Aloka Co. Ltd.), for $^{14}$C, respectively. Protein contents of cultured cells per dish were determined by the Lowry method (22).

### Collapsed BCEC Study

In order to examine the effect of medium osmolarity on the internalization of [$^{125}$I]ebiratide by cultured BCEC, the uptake experiment was also carried out in a hypertonic buffer (the incubation solution with 1.2 *M* sucrose, 1600 mOsm). The subsequent procedure was the same as described above.

### Effect of Endocytosis Inhibitors and a Metabolic Inhibitor on the Internalization of [$^{125}$I]Ebiratide

According to the previous study (6), cultured BCEC were preincubated at 37°C for 20 min in the incubation solution in the presence or absence of dansylcadaverine (500 μ*M*) or phenylarsine oxide (100 μ*M*), which are known as endocytosis inhibitors and 2,4-dinitrophenol (1 m*M*), which is a metabolic inhibitor. Phenylarsine oxide was dissolved in dimethyl sulfoxide (DMSO) to produce the concentration of 100 m*M* and was diluted with a 1000× volume of the incubation solution. The control experiment in the presence of 0.1% DMSO was also performed. The acid-resistant binding of [$^{125}$I]ebiratide was determined by the acid wash method described above.

### Data Calculation

According to Terasaki *et al.* (1), the data for the internalization can be expressed as the cell-to-medium concentration ratio, corrected for extracellular space using [$^{14}$C]sucrose, as follows.

For the acid-resistant binding,

$$\text{cell/medium (μl/mg of protein)} = [(^{125}\text{I-R} - {}^{125}\text{I-S} \times {}^{14}\text{C-R}/{}^{14}\text{C-S})/\text{mg of BCEC protein}]/[{}^{125}\text{I-M}/\mu\text{l of medium}] \quad (1)$$

where $^{125}$I-R and $^{125}$I-S, $^{14}$C-R and $^{14}$C-S are the $^{125}$I- and $^{14}$C-radioactivities in the acid-resistant and acid-soluble fraction associated with BCEC, respectively, and $^{125}$I-M is $^{125}$I-radioactivity in the incubation solution.

## RESULTS

### Time Course of the Acid-Resistant Binding of [$^{125}$I]Ebiratide

The time course of acid-resistant binding of [$^{125}$I]ebiratide to cultured monolayers of BCEC is illustrated in Fig. 1. The value expressed as cell/medium ratio of [$^{125}$I]ebiratide was corrected for an extracellular space, determined by using [$^{14}$C]sucrose. The acid-resistant binding of [$^{125}$I]ebiratide increased with increasing time, whereas only a slight time-dependent acid-resistant binding of [$^{14}$C]sucrose was observed over 120 min. The cell/medium ratio of the acid-resistant binding of [$^{125}$I]ebiratide was 1.65 ± 0.19 μl/mg of protein at 120 min.

### Stability of [$^{125}$I]Ebiratide

Figure 2 shows the intact percentage of [$^{125}$I]ebiratide in acid-resistant binding to cultured monolayers of BCEC analyzed by HPLC. At all of the examined incubation times, 24 to 39% of ebiratide was detected as the intact form of [$^{125}$I]-ebiratide.

Figure 3 shows the HPLC chromatograms of [$^{125}$I]ebiratide in the incubation solution (A) and acid-resistant fraction (B) after incubation with cultured monolayers of BCEC at 37°C for 60 min. As clearly seen from Fig. 3A, no metabolite of labeled ebiratide in the incubation solution was observed. On the other hand, the HPLC chromatogram of the acid-resistant fraction shows three peaks, and more than 30% of ebiratide was present in the intact form in the acid-resistant fraction applied to the HPLC system. In addition, as shown in Table I, acid-resistant binding of [$^{125}$I]ebiratide to BCEC was not inhibited by 10 m*M* histidine at 37°C for 60 min.

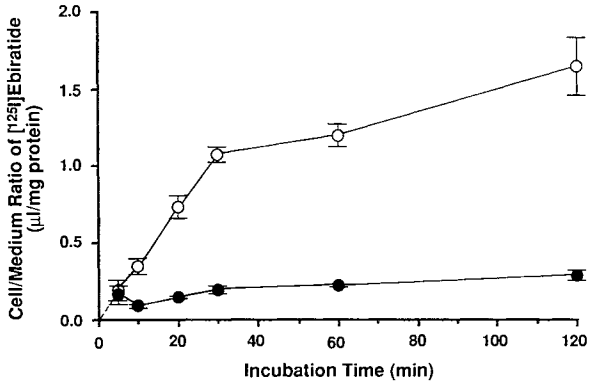


Fig. 1. Time courses of acid-resistant binding of [$^{125}$I]ebiratide (○) and [$^{14}$C]sucrose (●) to cultured monolayers of BCEC. After incubation of [$^{125}$I]ebiratide or [$^{14}$C]sucrose with cells at 37°C for 5–120 min, incubation solution was removed and the cells were incubated with 1 ml of ice-cold acetate–barbital buffer (pH 3.0) at 4°C for 10 min. Then acetate–barbital buffer was removed and radioactivity was counted as the acid-soluble fraction. After solubilization of cells, radioactivity was counted as the acid-resistant fraction. Cell-to-medium concentration ratio (cell/medium) was calculated using Eq. (1) in the text. Each point represents the mean ± SE of four experiments.

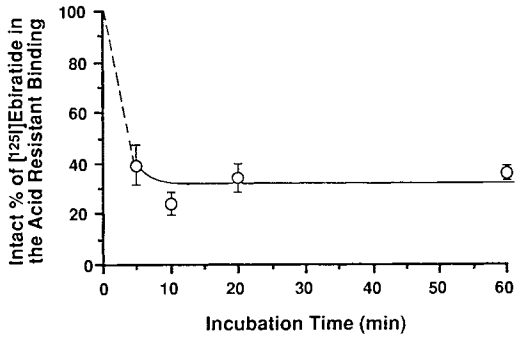


Fig. 2. Intact percentage of [$^{125}$I]ebiratide in the acid-resistant binding after incubation with cultured monolayers of BCEC. [$^{125}$I]Ebiratide was incubated for 5–60 min at 37°C and the acid-resistant binding of [$^{125}$I]ebiratide was analyzed by HPLC. Each point represents the mean ± SE of four experiments.

### Effect of Temperature Osmolarity, Endocytosis Inhibitors, and a Metabolic Inhibitor on the Acid-Resistant Binding of [$^{125}$I]Ebiratide

As shown in Table II, the incubation of [$^{125}$I]ebiratide with BCEC at 4°C for 60 min resulted in a decrease in acid-resistant binding to only 12% of the control value, showing a marked effect of temperature. When the osmolarity of the incubation solution was increased from 300 to 1600 mOsm under the same conditions, the acid-resistant binding of [$^{125}$I]ebiratide decreased significantly (Table II). In the ex-

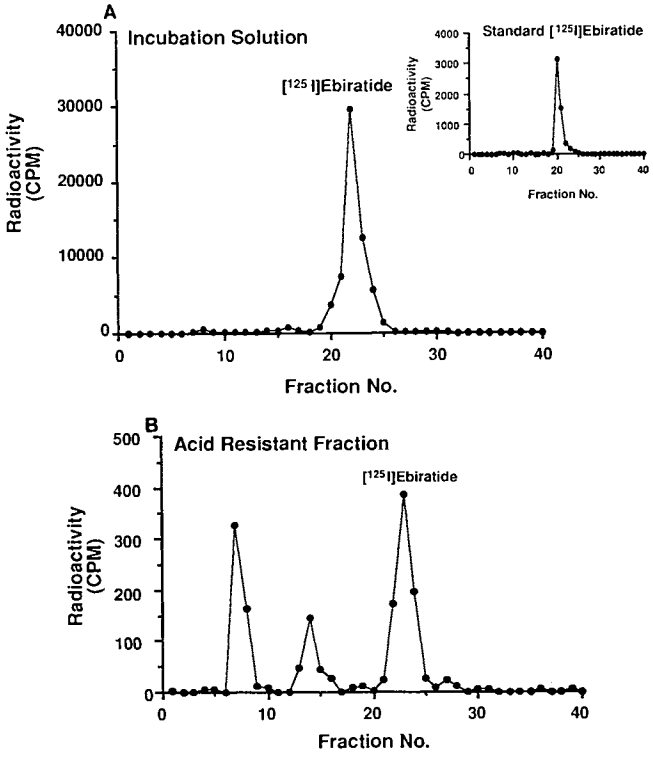


Fig. 3. HPLC chromatograms of [$^{125}$I]ebiratide in the incubation solution (A) and acid-resistant fraction (B) after incubation with cultured monolayers of BCEC at 37°C for 60 min. The HPLC analysis method is described under Materials and Methods. An HPLC chromatogram of a standard sample of [$^{125}$I]ebiratide is illustrated in the inset.

Table I. Effect of Histidine and Several Peptides on the Acid-Resistant Binding of [$^{125}$I]Ebiratide to Cultured Monolayers of BCEC

| Inhibitor | Concentration (m*M*) | Acid-resistant binding (% of control)[a] |
|---|---|---|
| Control | | 100 ± 4.0 |
| Histidine | 10.0 | 98.5 ± 5.5 |
| Poly-L-lysine | 0.30 | 29.6 ± 2.4** |
| Protamine | 0.30 | 12.4 ± 1.1** |
| Poly-L-glutamic acid | 0.30 | 95.5 ± 13.6 |
| Histone | 0.10 | 68.7 ± 5.7* |
| ACTH | 0.10 | 76.1 ± 5.4* |
| Insulin | 0.010 | 123 ± 21.0 |
| Transferrin | 0.010 | 188 ± 36.0 |

[a] Cells were incubated at 37°C for 60 min in the presence or absence of several inhibitors. Each value represents the percentage of control value (mean ± SE of three or four experiments).
* Significant inhibition ($P < 0.05$).
** Significant inhibition ($P < 0.001$).

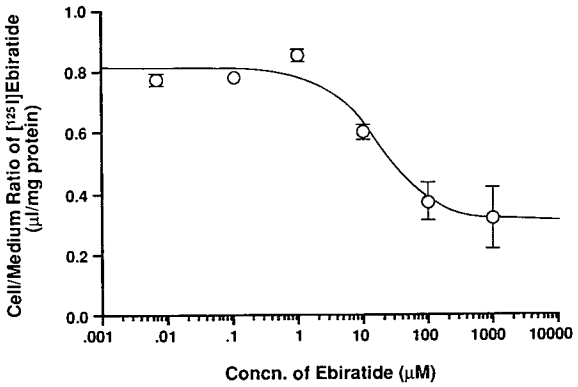


Fig. 4. Concentration dependence of the acid-resistant binding of [$^{125}$I]ebiratide to cultured monolayers of BCEC. [$^{125}$I]Ebiratide (7.14 n*M*) and unlabeled ebiratide at various concentrations (100 n*M*–1 m*M*) were incubated with BCEC at 37°C for 30 min. The acid-resistant binding (as cell/medium ratio) was determined as described in the legend to Fig. 1. Each point represents the mean of two or three experiments.

periments with endocytosis inhibitors, the acid-resistant binding was significantly decreased by dansylcadaverine (500 μ*M*) and phenylarsine oxide (100 μ*M*) (Table II). Moreover, the addition of 2,4-dinitrophenol (1 m*M*), an uncoupler of oxidative phosphorylation, diminished significantly the acid-resistant binding of [$^{125}$I]ebiratide.

### Concentration Dependence of the Acid-Resistant Binding of [$^{125}$I]Ebiratide

Figure 4 illustrates the dependence of acid-resistant binding of [$^{125}$I]ebiratide on the concentration of unlabeled ebiratide (100 n*M*–1 m*M*). This indicated that the internalization of [$^{125}$I]ebiratide by BCEC was saturable. By the nonlinear least-squares regression analysis of the data with a microcomputer program, MULTI (23), the maximal internalization capacity ($B_{max}$) at 30 min, the half-saturation constant ($K_d$), and the nonsaturable binding were estimated to be (mean ± SD) 7.96 ± 6.4 pmol/mg of protein, 15.9 ± 6.4 μ*M*, and 0.30 ± 0.03 μl/mg of protein, respectively.

Table II. Effect of Temperature, Osmolarity, Endocytosis Inhibitors, and a Metabolic Inhibitor on the Acid-Resistant Binding of [$^{125}$I]Ebiratide to Cultured Monolayers of BCEC

| Condition | Acid-resistant binding (% of control)[a] |
|---|---|
| Control | 100 ± 16.1 |
| Low temperature (4°C) | 11.8 ± 1.8** |
| Hypertonic (1600 mOsm) | 47.1 ± 3.6* |
| Dansylcadaverine (500 μ*M*) | 20.5 ± 4.9** |
| 2,4-Dinitrophenol (1.0 m*M*) | 42.3 ± 2.4* |
| Control | 100 ± 23.2[b] |
| Phenylarsine oxide (100 μ*M*) | 35.9 ± 6.1[b]* |

[a] After the preincubation of cells for 20 min under several conditions, [$^{125}$I]ebiratide was added to initiate the uptake. Acid-resistant binding was determined after the 60-min incubation. Each value represents the percentage of control value (mean ± SE of three or four experiments).
[b] As phenylarsine oxide was dissolved in 0.1% dimethyl sulfoxide (DMSO), the control experiment was performed in the presence of 0.1% DMSO.
* Significant inhibition ($P < 0.05$).
** Significant inhibition ($P < 0.005$).

### Effect of Several Peptides on the Acid-Resistant Binding of [$^{125}$I]Ebiratide

As shown in Table I, the acid-resistant binding of [$^{125}$I]ebiratide was markedly inhibited by polycationic peptides, poly-L-lysine (300 μ*M*) and protamine (300 μ*M*), but was not affected by a polyanionic peptide, poly-L-glutamic acid (300 μ*M*). The acid-resistant binding was not affected by insulin (10 μ*M*) but was inhibited significantly by histone (100 μ*M*) and ACTH (100 μ*M*). The acid-resistant binding was enhanced to 188% by transferrin (10 μ*M*).

## DISCUSSION

An absorptive-mediated endocytosis (or adsorptive endocytosis) system for the BBB transport of basic peptides has been proposed by the study of cationized BSA (7), cationized IgG (8), E-2078 (1), histone (9), and ebiratide (6) using isolated brain capillaries. Recent studies, however, have shown that BCEC are more useful to elucidate the transport mechanisms of peptides at the BBB *in vitro*. The advantages of using BCEC are that (i) transport mechanisms can be studied under the culture conditions, (ii) cell monolayers are highly viable, and (iii) cellular membranes can be kept polarized. For the delivery of centrally acting peptides such as ebiratide to brain, it is important that the peptide is transported through the luminal membrane of BBB, because this is the first step for delivery to the brain. Thus, in the present study, BCEC was employed to confirm the possible mechanism of ebiratide transport at the luminal side of BBB.

The acid-resistant binding of [$^{125}$I]ebiratide increased with time, while the cell/medium ratio of [$^{14}$C]sucrose used as extracellular marker showed a slight time dependency (Fig. 1). This slight time dependency for the cell/medium

ratio of [$^{14}$C]sucrose may be ascribed to the fluid-phase endocytosis of the capillary endothelial cells. Apparent temperature and osmolarity dependencies of acid-resistant binding of [$^{125}$I]ebiratide (Table II) suggest that ebiratide is significantly internalized into the intracellular space of BCEC. This mechanism of [$^{125}$I]ebiratide taken up by BCEC was supported by the finding that internalization of [$^{125}$I]ebiratide was significantly inhibited by dansylcadaverine and phenylarsine oxide. Dansylcadaverine is known to inhibit the internalization of epidermal growth factor by inhibiting transglutaminase activity in cell membrane and resulting the suppression of the coated pit formation (24). Phenylarsine oxide is known to inhibit the internalization of insulin (25) and epidermal growth factor (26) by the interaction with the SH group in the cell membrane. Interestingly, the effect of phenylarsine oxide on the acid-resistant binding of [$^{125}$I]ebiratide to BCEC was different from that reported with ebiratide using isolated brain capillaries (6). One possible reason for the discrepancy of the effect of phenylarsine oxide might be that EDTA, which was added to inhibit the enzymatic metabolism in the uptake study using isolated brain capillaries (6), diminished the effect of phenylarsine oxide as an SH blocker. A significant inhibitory effect by metabolic inhibitor, 2,4-dinitrophenol, also supports that ebiratide is taken up by endocytosis system which is known to be energy dependent.

As shown in Fig. 4, the apparent concentration dependency of acid resistant binding of [$^{125}$I]ebiratide suggests that the specific endocytosis system for ebiratide exists on the surface of BCEC. The $K_d$ value (15.9 $\mu M$) in this study is in good agreement with the $K_d$ value determined using isolated brain capillaries for E-2078 (4.62 $\mu M$) (1) and for ebiratide (62.1 $\mu M$) (6). The value is also in good agreement with the value of histone (15.2 $\mu M$) (9), while higher than those of cationized BSA (0.8 $\mu M$) (7) and cationized IgG (0.90 $\mu M$) (8), which are transported to BBB via an absorptive-mediated endocytosis. On the other hand, the $K_d$ value of ebiratide is about 7000–40,000 times greater than the values for compounds transported via receptor-mediated endocytosis (atrial natriuretic factor, insulin, transferrin) (27–29). Therefore, it is supposed that ebiratide is internalized in BCEC via an absorptive-mediated endocytosis. As the *in vivo* levels of ebiratide in rat and human plasma are less than 100 n$M$ (30), no saturation seems to occur in the transport process of ebiratide at the BBB *in vivo*.

Moreover, to clarify that ebiratide is transported into cultured BCEC via nonspecific absorptive-mediated endocytosis, inhibition studies with several peptides were also carried out. Ebiratide contains basic amino acids, such as histidine and lysine, and an octylamino group in its structure, and its isoelectric point (pI) is 10 (6). Thus, the peptide is positively charged at the physiological pH of 7.4. On the other hand, it is well-known that the endothelial cell membrane has anionic sites (31). Therefore, it was presumed that the basicity of ebiratide plays an important role in its internalization in BCEC. As shown in Table I, remarkably different effects between polycations (poly-L-lysine and protamine) and a polyanion (poly-L-glutamic acid) on the acid-resistant binding of [$^{125}$I]ebiratide were observed. These characteristics are similar to our recent results obtained using isolated brain capillaries (E-2078 and ebiratide) (1,6). Moreover, the acid-resistant binding was significantly inhibited by histone, which is transported through the BBB via an absorptive-mediated endocytosis as a basic peptide (9). These results suggest that ebiratide is transported by an absorptive-mediated endocytosis into BCEC. Interestingly, the acid-resistant binding was also inhibited by ACTH, having a pI value of 8.5 (32) (Table I). The result suggests that ACTH may be also internalized via the same system of ebiratide, i.e., AME, due to the basicity of ACTH. On the other hand, no inhibitory effect of insulin and transferrin (Table I), for which specific receptors are known to exist in brain capillaries (28,29,33), discriminates the possibility that ebiratide is transported via a receptor-mediated endocytosis using insulin and transferrin receptors.

As shown in Figs. 2 and 3B, the acid-resistant fraction of [$^{125}$I]ebiratide is obviously degraded. With respect to the degradation of peptide during transport into cells, at least three possibilities should be considered. The peptide is (i) transported into the cells as the intact form and then metabolized in the intracellular space, (ii) degraded to $^{125}$I-labeled amino acid, i.e., [$^{125}$I]histidine, at the surface of the plasma membrane of the cells and [$^{125}$I]histidine is transported via a carrier-mediated system for the amino acid, and (iii) degraded to $^{125}$I-labeled shorter peptides at the surface of the plasma membrane of the cells and these peptide fragments of ebiratide are transported *via* a certain transport mechanism. No significant inhibitory effect of 10 m$M$ histidine to acid-resistant binding of [$^{125}$I]ebiratide (Table I) suggests that $^{125}$I-radioactivity of the acid-resistant fraction could not be ascribed to the transport of [$^{125}$I]histidine. Furthermore, no metabolism of the labeled ebiratide occurred in the incubation solution with cultured BCEC (Fig. 3A), indicating that ebiratide is stable to peptidases associated with the BCEC luminal membrane. So possibilities (ii) and (iii) should be ruled out. Therefore, [$^{125}$I]ebiratide should be internalized via endocytosis in BCEC as an intact form and then degraded by intracellular enzymes to some extent.

Though the physiological relevance of an absorptive-mediated endocytosis at the blood–brain barrier has not been clarified yet, the system has been demonstrated to be a promising pathway for the delivery of the several kinds of basic peptides into the brain (1,2,6–9). One of the advantages of utilize the system would be that it does not require the strict primary structure of the peptide. As the basicity of the peptide would be the most determinant factor for the peptide association to the surface of the capillary endothelial cells, basic amino acids such as lysine and arginine would be very important to increase the availability from the systemic circulation into the brain.

In conclusion, it is confirmed by the present study using BCEC that a novel ACTH analogue, ebiratide, is transported at the BBB via an absorptive-mediated endocytosis system. Chemical modification to obtain a stable and basic peptide should be a promising strategy to convert a native physiologically active peptide into a neuropharmaceutical.

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