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Extracellular Biotransformation of β-Endorphin in Rat Striatum and Cerebrospinal Fluid

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ABSTRACT

Numerous studies have investigated the behavioural effects of β-endorphin, both endogenous and exogenously applied. However, the potential for biotransformation of β-endorphin in the extracellular space of the brain has not been previously directly addressed *in vivo*. Utilising microinfusion/microdialysis and MALDI mass spectrometry, we investigated β-endorphin biotransformation in the striatum rats. We infused 1.0 nmol β-endorphin into the striatum of adult male Fischer rats and observed rapid cleavage resulting in β-endorphin 1-18, as well as several fragments resulting from further N-terminal degradation. *In vitro* studies with incubation of full-length β-endorphin, with and without protease inhibitors, in the incubation fluid of isolated striatal slices indicate β-endorphin is initially cleaved predominantly at the Phe^{18}-Lys^{19}, position, as well as at the Leu^{17}-Phe^{18} position. Investigations of cerebrospinal fluid revealed similar enzymatic cleavage of β-endorphin. The observed pattern of cleavage sites (Phe^{18}-Lys^{19} and Leu^{17}-Phe^{18}) is consistent with published *in vitro* studies of purified insulin-degrading enzyme cleavage of β-endorphin. The binding affinities of full-length β-endorphin, as well as previously identified β-endorphin fragments α-endorphin (β-endorphin 1-16) and γ-endorphin (β-endorphin 1-17), and the fragment identified in the current studies, β-endorphin 1-18, at heterologously expressed MOP, DOP, and KOP receptors (μ, δ, and κ-opioid receptors, respectively) were determined; the affinity of the truncation fragments is reduced at each of the receptors compared to the affinity of full length β-endorphin.

INTRODUCTION

β-Endorphin is an endogenous opioid peptide, and has an important role in nociception, locomotor activity, and reward, as well as other behavioural phenomena (1). Generally neuropeptides are susceptible to extracellular peptidases, which can potentially result in peptide fragments with different bioactivity (2,3). For instance, angiotensin converting enzyme (ACE) is a well known protease, known to cleave angiotensin peptides, resulting in fragments with differential bioactivity (4). Neprilysin (NEP), initially named enkephalinase as it was discovered to be the enzyme responsible for terminating the activity of enkephalins following extracellular release, is known to be involved in the extracellular proteolysis of a number of peptides (4). Following their discovery and characterisation, these proteases have served as important targets for therapeutics, notably in hypertension, as well as in other disorders.

Shortly after the discovery of β-endorphin (5), *in vitro* studies showed β-endorphin is capable of being cleaved via proteolytic enzymes, in striatal slices (6) and synaptic membrane preparations (7,8). These studies used chromatographic techniques to identify the products, which included α- and γ-endorphin (β-endorphin 1-16 and 1-17, respectively), which had previously been identified in hypothalamic extracts (9). A number of studies using synthetic exogenous α- and γ-endorphin, as well as fragments of these peptides, showed behavioural effects which in many cases differ from those induced by full-length β-endorphin (Reviewed in 10 and 11). Since the time of these studies, advances in biological mass spectrometry have resulted in specificity of peptide identification which surpasses that obtainable via chromatography. Additionally, advances in the microdialysis sampling of extracellular fluid of the brain allows for *in vivo* investigation of proteolysis. In the current studies, we have applied

these methodological advancements to the investigation of β-endorphin biotransformation *in vivo*.

Recent studies using microdialysis and ELISA for detection of β-endorphin-like immunoreactivity have suggested that β-endorphin is released into the extracellular space of the ventral striatum (12-14). Note, these studies were performed using commercially available antibodies, which show cross-reactivity with α-endorphin and γ-endorphin (according to cross-reactivity information provided by the vendor). In general, lacking *a priori* knowledge about the identity of the proteolytic products for a neuropeptide, methods relying on antibodies are not well-suited to the study of extracellular proteolysis of the neuropeptide. By contrast, microdialysis recovery of peptide fragments formed *in vivo*, coupled with mass spectrometry for detection and identification of these fragments, is a flexible, sensitive, and specific method for addressing the question of extracellular proteolysis of neuropeptides (15,16).

The current study was designed to determine whether β-endorphin, *in vivo*, is subject to extracellular proteolysis in the central nervous system, i.e. whether there is an endorphinase-like protease, analogous to enkephalinase. To address this question, we determined the products of extracellular processing of β-endorphin in the rat striatum using methodology that we previously developed and applied to *in vivo* dynorphin A [1-17] extracellular processing in the rat striatum, utilising microinfusion/microdialysis and MALDI mass spectrometry (16). Based on our present results, indicating that β-endorphin *does* undergo extracellular proteolysis, we have addressed two corollary questions: 1. which protease(s) is responsible? *In vitro* studies using striatal slices and CSF were conducted to partially characterise the enzyme(s) involved in β-endorphin extracellular processing. 2. do the resulting proteolytic products show binding affinity to the

opioid receptors? The binding affinities for the μ-, δ-, and κ-opioid receptors of the major N-terminal fragments of β-endorphin observed in these studies were determined.

MATERIALS AND METHODS

Materials

Rat and human β-endorphin (β-endorphin-r and β-endorphin-h, respectively), α-endorphin, and γ-endorphin were obtained in HPLC-purified form from Peninsula Laboratories (San Carlos, CA, USA). β-Endorphin 1-18 was synthesised and HPLC purified by The Rockefeller University Proteomics Resource Centre. Model IBR guide cannulae for microdialysis with accompanying probes were obtained from Bioanalytical Systems (West Lafayette, IN, USA). Anti-insulin-degrading enzyme rabbit polyclonal antibody was from Chemicon (Temecula, CA, USA), and anti-insulin-degrading enzyme monoclonal antibody 9B12 was obtained from Covance Research Products (Denver, PA, USA). Horseradish peroxidase conjugated goat anti-rabbit secondary antibody, metal enhanced diaminobenzidine (DAB), and Protein G sepharose beads were obtained from Pierce Biotechnology (Rockford, IL, USA). POROS 50 R2 beads were obtained from PerSeptive Biosystems (Framingham, MA, USA). Eppendorf 10 μl gel loading tips were purchased from Brinkmann Instruments (Westbury, NY, USA). [^{3}H][D-Ala^2, N-Me, Phe^4, glycinol^5]enkephalin (DAMGO) (64.0 Ci/mmol) and [^{3}H]U69,593 (55.0 Ci/mmol) were purchased GE Health Care/Amersham Biosciences (Piscataway, NJ, USA). [^{3}H]Naltrindole (35.0 Ci/mmol) and was purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). EcoScint A was purchased from National Diagnostic, Inc. (Atlanta, GA, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

Animals

Fischer rats were used in all studies described herein (male, weight between 175 and 250 g, age 13-14 weeks; Charles River Laboratories, Wilmington, MA). Animals were individually caged, given free access to food and water, and kept in temperature (25 ºC) and light controlled rooms. All animal care and experimental procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and approved by the Institutional Animal Care and Use Committee of The Rockefeller University.

Microdialysis

Surgery was performed on male Fisher rats in a similar manner to that previously described (9). Briefly, rats were anaesthetised with ketamine/xylazine prior to stereotaxic surgery. The combination microinfusion/microdialysis probes were placed in the guide cannulae and directly implanted during surgery (Bregma coordinates: A=+0.5 mm, L = - 3.0 mm, V= -5.2 mm). The guide cannulae are made of inert plastic and are 1 mm in diameter. The combination probes are composed of polyacrylonitrile membrane with a molecular weight cut-off of 30 kDa, are 2 mm in length and 320 μm in diameter; a central infusion cannula composed of fused silica extends just beyond microdialysis membrane (total volume of 0.2 μl). The rats were placed directly in the microdialysis chambers (Med-Associates, Georgia, VT, USA) following surgery and allowed to acclimatise for 24 h. Approximately 36-48 h elapsed between surgery and the beginning of the experiment. The night before the experiment, a syringe pump was used to pump perfusion fluid (150 mM NaCl; 4 mM KCl; 2.3 mM $CaCl_2$; 1.0 mM $MgCl_2$, pH 6) through the microdialysis probe overnight (0.3 μl/minute). The rate of perfusion was then increased to 1.0 μl/minute for 1

hour, after which we collected two 20-minute samples (20 µl) for baseline controls. β-Endorphin-r was dissolved at a concentration of 0.5 mM in perfusion fluid, and 2 µl was infused via the central microinfusion cannula at a rate of 10 µl/minute. Samples (20-minute durations) were continuously collected following β-endorphin infusion.

Striatal Slice

For *in vitro* enzymatic breakdown in striatal slice preparations, following brief exposure to carbon dioxide, rats were decapitated in a stress-minimised environment. The brains were placed in a matrix and sliced; the striatum was dissected out from a 2 mm slice, while on ice, and placed on dry ice. The striatal slices were thawed by incubation with phosphate buffered saline (PBS, pH 7.2; 85 µl) for 5 min. The slices were then incubated for an additional 5 min with PBS (85 µl). Aliquots of the incubation fluid (20 µl) were combined with the appropriate concentration of protease inhibitors and β-endorphin-r and incubated at 37 °C. Fractions of 1.0 µl were removed at various time-points for mass spectral analysis.

Cerebrospinal Fluid

For cerebrospinal fluid analyses, rats were briefly anaesthetised with carbon dioxide, and a 25 gauge needle was inserted 5-6 mm into the cisterna magna. Approximately 100 µl was withdrawn and immediately frozen. Immediately prior to the processing experiments, CSF was thawed and separated into 20 µl aliquots. Appropriate concentrations of protease inhibitors were added, where applicable, and the aliquots were incubated at 37 °C with β-endorphin-r. Fractions of 1.0 µl were removed at various time-points for mass spectral analysis, as described below.

Sample Preparation

Samples were prepared for matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry as previously described (16). Briefly, an external standard, 25 µl of 10 nM thymopoietin fragment II peptide (molecular weight 1611), was added directly to the samples (20 µl for microdialysis, 1.0 µl for striatal slice preparations and cerebrospinal fluid). Next, 2 µl of 25% slurry of POROS 50 R2 beads in 50% MeOH, was added to bind peptides present in the fractions. The mixtures were vortexed and placed in Eppendorf 10 µl gel loading tips that were pulled and cut to act as a frit. A 1 ml syringe was used to pack the beads. These beads with bound peptides were then washed with 50 µl of 0.1% trifluoroacetic acid. Next the bound peptides were eluted directly onto the Voyager DE-STR (Applied Biosystems, Foster City, CA, USA) sample plate for MALDI time-of-flight mass spectrometric analysis using 1 µl 2:1 acetonitrile: water (0.1% trifluoroacetic acid), saturated with α-cyano-4-hydroxycinnamic acid as the MALDI matrix.

MALDI Mass Spectrometry

For MALDI time-of-flight mass spectrometric analysis, a Voyager-DE STR Mass Spectrometer was used in both linear and reflectron modes, with delayed extraction. This instrument is configured with a nitrogen laser (337 nm), delivering 3 Hz pulses. Each spectrum represents a sum of 100 laser shots, and has been smoothed, calibrated, and analysed using the programme M-over-Z (Genomic Solutions, Lansing, MI, USA), with peptide identification using the programme PAWS (Genomic Solutions). The identities of peptides were confirmed using partial sequencing via tandem mass spectrometry, with samples prepared as previously (16). For tandem mass spectrometry experiments, we employed a MALDI ion trap mass spectrometer (17) that

incorporated a commercial ion trap mass analyser (Thermo Finnigan LCQ Classic, San Jose, CA, USA). The configuration and settings of the instrument for tandem mass spectrometry of peptides have previously been described (17).

Immunoprecipitation

Immunoprecipitations were carried out using anti-IDE monoclonal antibody 9B12 (18). The antibody was added at a concentration of 10 μg/ml to CSF (100μl). Protein G sepharose beads were then added and the samples were incubated at 4 °C for 2h. The supernatant was removed following centrifugation (10,500 x g), and the beads were washed 4 times with ice-cold PBS and resuspended with PBS (100μl). β-endorphin-r was then added, in parallel, to untreated CSF, the supernatant fraction, and the resuspended beads, and incubated at 37°C. At various time intervals, 1 μl aliquots (1% of the initial volume) were removed for analysis by mass spectrometry.

Immunoblotting

Immunoblots were performed according to standard procedures. The dissected striata (right and left) were combined and homogenised using sonication in 1 mL PBS. The homogenised tissue was diluted 6-fold with 6x SDS sample buffer, containing SDS and dithiothreitol for denaturation and reduction. The tissue was then separated via one-dimensional SDS-PAGE and transferred to a PVDF membrane via semi-dry electroblotting. The membrane was then incubated with a commercially available anti-IDE rabbit polyclonal antibody (1:2000), followed by horseradish peroxidase conjugated goat anti-rabbit antibody (1:2000). The membrane was visualised by addition of enhanced DAB.

Cell Culture

Chinese hamster ovary (CHO) cells were stably transfected with either the human δ opioid receptor (hDOP-CHO), the κ opioid receptor (hKOP-CHO) or the μ opioid receptor (hMOP-CHO). The hDOP-CHO and the hKOP-CHO cells were obtained from Dr. Larry Toll (SRI International, Palo Alto, CA USA). The hMOP-CHO cells were obtained from Dr. George Uhl (NIDA Intramural Programme, Baltimore, MD USA). The cells were grown in 100 mm dishes in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin (10,000 units/mL) at 37 ºC in a 5% CO_2 atmosphere.

Radioligand Binding

CHO cell membranes were prepared as previously described (19). The radioligands used were [^{3}H]DAMGO (μ), [^{3}H]naltrindole (δ), and [^{3}H]U69,593 (κ). Binding was performed in 50 mM Tris-HCl, pH 7.5, at 25 ºC for 60 min or at 4^{o}C for 2 h for [^{3}H]DAMGO and [^{3}H]U69,593 and 3 hr at 25^{o}C or 4^{o}C for [^{3}H]naltrindole. Membrane protein (50 μg hMOP-CHO, 20 μg hDOP-CHO and 100 μg hKOP-CHO) was incubated with radiolabelled ligand in the presence of varying concentrations of the opioid peptides. Nonspecific binding was determined in the presence of 10 μM naloxone. Incubations were performed in triplicate at a final volume of 1 ml of 50 mM Tris-HCl, pH 7.5. To determine the effect of endogenous proteases associated with the membrane preparations, protease inhibitors were included in the binding assay. The protease inhibitors, 10 μM captopril, 30 μM bestatin, and 100 μM phenyl methyl-sulfonyl fluoride (PMSF) were included. The protease inhibitors, bacitracin at 50 μg/ml and 3 mM $MgCl_2$ inhibited the binding of the μ-selective peptide [^{3}H]DAMGO to membranes. Therefore,

bacitracin and $MgCl_2$ were not included in the protease inhibitor cocktail. The binding reaction was terminated via rapid filtration though Schleicher & Schuell No. 32 glass fibre filters using a 48-well Brandel cell harvester. For [^{3}H]U69,593 and [^{3}H]naltrindole binding, filters were presoaked in 0.25% polyethyleneimine for 60 min. After filtering the samples, filters were rinsed three times with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.5 and subsequently counted in 2 ml of EcoScint A scintillation fluid for 2 min.

RESULTS

In Vivo Microinfusion/Microdialysis Study. Figure 1 shows representative mass spectra following infusion of 1.0 nmol β-endorphin-r into a single rat. The peaks observed correspond to the masses of the following fragments: β-endorphin (1-18), (2-18), (3-18), (4-18), (5-18), (6-18), (8-18), (19-31), (20-31) (20-29), and (20-28). Note, the peak labelled (20-31) has a measured *m/z* 1349.8, which could theoretically be attributed to (7-18) [calculated monoisotopic *m/z* 1349.731], (19-30) [1349.838], or (20-31) [1349.801]. Using MALDI-ion trap tandem mass spectrometry, the identity of this peak was unequivocally determined to result from (20-31) (Figure 2). In addition to the 1349.8 *m/z* peak, tandem mass spectra for the 2006.0, 1842.9, and 1477.9 definitively indicate these peaks correspond to β-endorphin 1-18, 2-18, and 19-31, respectively (Figure 2). Although not all peaks (Figure 1) were investigated by tandem mass spectrometry, the peaks are labelled based on the corresponding masses of the β-endorphin fragments, within 200 ppm, as well as absence from the baseline (i.e. they only appear following full-length β-endorphin infusion).

The spectra shown in Figure 1 correspond to fragments recovered in three consecutive 20-minute intervals. The initial fragments of highest peak intensity, (1-18) and (2-18), show a relative decrease in peak intensity, as a function of time, while further truncation fragments, (5-18), (6-18), and (8-18), increase in relative intensity. The C-terminal complement peptide of (1-18), namely (19-31), has relatively low intensity, apparently partially due to aminopeptidase activity resulting in (20-31). In addition, a carboxypeptidase or an endopeptidase, perhaps specific for basic residues, likely effects C-terminal truncation of (20-31) to yield (20-28) and (20-29). The experiment was repeated on five separate rats, and the fragments identified were generally reproducible, although the relative magnitude of the observed peaks differed somewhat.

Striatum Slice Studies. Figure 3A shows the results for β-endorphin-r degradation in the incubation fluid from striatal slices. Dissected striata (both left and right) from a single rat were pooled and washed with 100 µl PBS for 5 min to remove cellular debris, which may result from damage incurred by dissection. The striata were then incubated a second time for 5 min; the incubation fluid was removed, aliquotted into 20 µl fractions, and the indicated concentrations of β-endorphin and protease inhibitor were added to the incubation fluid. The incubation fluid was used, rather than simply adding the peptide in the presence of the slices, to prevent the possibility of the peptide entering the cells of the tissue (whether by passive diffusion or by active transport) and being metabolised by intracellular enzymes, followed by the products in turn exiting the tissue into the sampled media. In the absence of protease inhibitors, 5 µM β-endorphin-r yielded the following fragments: (1-18), (2-18), (2-17), (19-31), and (20-31) (Figure 3A, top panel). Bestatin, an aminopeptidase inhibitor, was added and prevented formation of (20-31) and (2-18),

and additional peptide fragments (1-17) and (18-31) were detected, consistent with the initial cleavage of β-endorphin-r at positions Phe^{18}-Lys^{19} and Leu^{17}-Phe^{18} (Figure 3A, second panel). In the presence of 1 mM 1,10-phenanthroline, a metalloprotease inhibitor, cleavage of β-endorphin-r into fragments was retarded (Figure 3A, third panel). All cleavage was prevented when the concentration of 1,10-phenanthroline was increased to 5 mM (Figure 3A, bottom panel).

Using a commercially available anti-IDE polyclonal antibody, immunoblotting was used to determine whether IDE is present in the striatum (Figure 3B). The presence of a band at the expected molecular mass (~110 kDa) indicates that IDE is present in the striatum. A similar band was observed using monoclonal antibody 9B12 (data not shown). Lower molecular weight bands (< 60 kDa) are clearly visualised, with a very strong band at ~15 kDa. These may represent degradation products of the IDE, or possibly non-specific recognition by the antibody. Attempts to detect IDE by immunoblotting in the striatal incubation fluid were not successful (data not shown), most likely due to the low overall levels of this enzyme which diffuse into the incubation fluid.

In Vitro *Cerebrospinal Fluid Study.* The results of β-endorphin-r incubation with rat CSF are shown in Figure 4A. β-endorphin(1-17), (1-18), (18-31), and (19-31) were observed as early as 15 min following incubation in CSF. The spectra shown correspond to a 4.5-hour incubation, where no further proteolytic fragments were observed compared to 15 min, indicating that the initially produced fragments are not subject to significant further proteolysis in CSF. The aminopeptidase inhibitor bestatin had no effect on the observation of any of the fragments, ruling out the possibility that β-endorphin (19-31) resulted from aminopeptidase activity on β-endorphin (18-31). We also tested the inhibitory potential of 1,10-phenanthroline, a metal

chelator which inhibits the action of most metalloproteases. At a concentration of 1 mM, this compound attenuated to a great degree the enzyme activity resulting in β-endorphin (1-17), (1-18), (18-31), and (19-31).

To determine whether IDE was responsible for the observed cleavage pattern, immunoprecipitation using a monoclonal antibody to IDE was performed (figure 4B). The antibody immunoprecipitates the enzymatic activity resulting in β-endorphin-r cleavage to 1-18/19-31 and 1-17/18-31 (bottom spectrum). The protein A/G sepharose beads used to effect the immunoprecipitation have an apparent affinity for full-length β-endorphin-r, which was not observed in the mass spectra of the immunoprecipitated fraction (this was also the case in mock immunoprecipitated CSF samples, to which no monoclonal antibody was added; data not shown). It is noteworthy that synthetic β-endorphin (1-18), when added to mock immunoprecipitated CSF samples, did not adhere to the protein A/G sepharose beads, consistent with the presence of β-endorphin (1-18) and the absence of full-length β-endorphin-r in the immunoprecipitated fraction (bottom spectrum). The immunoprecipitation did not result in complete immunodepletion of the enzymatic activity (middle-spectrum, CSF supernatant).

Opioid Receptor Binding Assays. The predominant fragment resulting from β-endorphin biotransformation *in vivo* which is predicted to maintain opioidergic activity is β-endorphin 1-18. We determined the apparent affinities for this fragment, as well as the additional fragment observed *in vitro*, γ-endorphin (β-endorphin 1-17), for inhibition of binding of selective radioactive ligands to the cloned μ-, δ-, and κ-opioid receptors (Table 1). The affinities of the fragments for each of the opioid receptors were reduced significantly compared to those for full-length β-endorphin, although the overall selectivity for μ- and δ-opioid receptors versus the κ-

opioid receptor is maintained. Note, in membranes prepared from CHO cells, we investigated the question of whether full-length β-endorphin1-31 and β-endorphin 1-18 are subject to degradation, using mass spectrometry. Our findings indicated that β-endorphin 1-31 is stable, while β-endorphin 1-18 is subject to a small degree of degradation to the nonopioidergic fragments, β-endorphin 2-18 and 4-18 (data not shown). Certain protease inhibitors which may have proved effective in preventing β-endorphin 1-18 degradation, such as bacitracin and 1,10-phenanthroline, were not used in the receptor binding assays because they directly inhibited opioid binding.

DISCUSSION

In the present study, we initially investigated the extracellular processing of β-endorphin-r in the rat dorsal striatum, utilising microinfusion/microdialysis coupled with MALDI mass spectrometry (Figure 1). The striatum was targeted both for technical reasons, as we investigated this region in our earlier study with dynorphin (16), and because β-endorphin-like immunoreactivity has been identified in the ventral striatum (12-14). The recovery of peptides via microdialysis is typically in the range of 1 to 10%, with generally decreasing recovery as a function of increasing peptide length and/or hydrophobicity. The differences in recovery of closely related biotransformation products is therefore expected to be small. The extent of recovery is also dependent on the microdialysis probe length, diameter, membrane composition, molecular weight cut-off, and perfusion fluid flow rate (as reviewed in 20). Because of the relatively modest recovery of β-endorphin, we infused somewhat high levels of β-endorphin (2 nmol), to would allow for detection of products even with very low recovery. Indeed, the

detection limits of MALDI-time-of-flight mass spectrometry under the present conditions is on the order of 2 fmol.

The time course of the *in vivo* recovery of β-endorphin biotransformation products indicates that the primary extracellular processing event involves cleavage at the Phe^{18}-Lys^{19} position. Subsequent processing likely involves aminopeptidase and carboxypeptidase cleavage of the initial truncation fragments. It is possible that collecting shorter time intervals would yield an improved understanding of the dynamics of the processing events, but it is unlikely any additional fragments would be observed, as the 20 minute fractions analysed are essentially the integral of shorter interval fractions. Of the observed fragments in this *in vivo* study, only (1-18) maintains the opioidergic sequence YGGFM necessary to confer binding and activity at opioid receptors (21). Our *in vivo* observation of β-endorphin 1-18 is novel, and contrasts to that which would be expected based on previous pioneering studies of *in vitro* β-endorphin biotransformation (6-8).

Given that we observed *in vivo* extracellular β-endorphin degradation, we wanted to determine the enzyme responsible, with initial screening of various enzyme inhibitors to screen the nature of the enzyme. We checked to see whether we might be able to conduct such studies using striatal slices, as this would allow for higher throughput, as well as necessitate using fewer animals. We observed a similar profile in the incubation fluid of striatal slices as we observed in the *in vivo* studies (Figure 2A). The observed fragments are consistent with the inference from our *in vivo* studies that the initial cleavage event is at the Phe^{18}-Lys^{19} position. Previous studies of β-endorphin metabolism in rat striatal slices, comparing gel filtration elution times of standards with the metabolites of [^{125}I]-porcine-β-endorphin, have indicated that the major metabolites of β-endorphin include γ-endorphin, α-endorphin and Met-enkephalin, with no

observation of β-endorphin (1-18) (22), in contrast to our findings in striatal slice preparations (Figure 2A). The reason for this discrepancy is not clear, although possible anomalies in gel filtration elution profiles cannot be ruled out. Additionally, differences in the sequence of porcine and rat β-endorphin (Figure 5) may have effects on the enzyme susceptibility, resulting in the differences in observations between the studies.

As the profile of β-endorphin cleavage was similar in the striatal slices, we screened protease inhibitors to determine the enzymes responsible. Incubation with the aminopeptidase inhibitor bestatin prevented the observation of (2-18), (2-17), and (20-31), and allowed for the observation of (1-17) and (18-31), indicating that there is also a cleavage event at the Leu^{17}-Phe^{18} position. It should be noted that when a mammalian protease inhibitor "cocktail" (Sigma) was used, resulting in final concentrations of 520 μM AEBSF, 7.5 μM pepstatin A, 7 μM E-64, 10 μM leupeptin, 0.04 μM aprotinin, and 20 μM bestatin, the β-endorphin degradation profile was nearly identical to that observed with 100 μM bestatin (data not shown), indicating that the responsible protease is likely not in the class of serine proteases (inhibited by AEBSF and aprotinin), aspartyl proteases (inhibited by pepstatin), or cysteine proteases (inhibited by E-64 and leupeptin). An enzyme activity, associated with synaptosomal membranes, was previously found to be capable of cleaving β-endorphin-h into γ-endorphin (β-endorphin 1-17), and hence named γ-endorphin-generating enzyme (23). Later, a similar enzyme activity against β-endorphin-h was detected in an EL-4 thymoma cell line (24), and subsequent characterisation revealed it to result from IDE (25). IDE was shown to cleave β-endorphin-h at the Leu^{17}-Phe^{18} and Phe^{18}-Lys^{19} sites, at approximately equal rates. Various cells of the immune system have been shown to contain IDE, cleaving exogenous β-endorphin (23,24).

A number of studies of IDE degradation of insulin have demonstrated a role for this enzyme in intracellular activity (reviewed in 26), and there is controversy over the possibility that IDE is active extracellularly, given that it lacks a secretory signalling sequence (see 27). However, there is evidence that IDE may also be active extracellularly. Immunohistochemical studies of postmortem brains from normal and Alzheimer's disease patients indicate that the majority of IDE is present in neurones intracellularly (28). In the brains from Alzheimer's disease patients, some senile neuritic plaques showed punctuate immunoreactivity, indicating an extracellular localisation for this enzyme, but functionality of plaque-associated IDE was not demonstrated (28). IDE has been shown to be released by microglial cells in culture (29) and is present in human cerebrospinal fluid (30), as well as on membranes of cultured cortical rat neurones (31), but it has not been shown to be active in the extracellular fluid of the brain. Our results are consistent with the notion that IDE can, in fact, be active extracellularly.

In our striatal incubation fluid studies, there is a difference in the levels of observed products resulting from cleavage of Leu^{17}-Phe^{18} (1-17/18-31) compared with those of Phe^{18}-Lys^{19} cleavage (1-18/19-31), with higher levels of the latter (Figure 2A). This difference is much greater than that observed for β-endorphin-r degradation in the CSF (Figure 3A), as well as that previously reported for β-endorphin-h with purified insulin-degrading enzyme (25). It is of note that the sequences of human and rat β-endorphin differ in three positions close to the C-terminus (Figure 4); these positions are not immediately adjacent to the sites cleaved by IDE, and their effect on β-endorphin susceptibility to IDE activity is not known. It is possible that (1-17) and (18-31) are more susceptible than (1-18) and (19-31) to further degradation by other enzymes that are also present in the striatum. It is also possible that additional enzymes are involved.

Our results here are consistent with a role for IDE in β-endorphin biotransformation in the striatum (Figure 2A). Immunoblotting, showing the localisation of IDE in the striatum (Figure 2B), further supports this interpretation. While the data shown here was collected for the dorsal striatum, other brain regions may show differences in the time-course of extracellular β-endorphin biotransformation. Such differences would reflect differences in relative extracellular concentrations of the enzymes involved in both the primary cleavage events, as well as secondary cleavage events, such as amino-terminal degradation. Indeed, studies have shown regional differences in levels of insulin degrading enzyme (32) and aminopeptidases (33). In addition to the studies discussed, we undertook preliminary slice incubation studies of various other brain regions (including the hypothalamus, hippocampus, amygdala, frontal cortex, and ventral striatum). Although we observed quantitative differences in the time-course of identified products, there were no qualitative differences in the primary cleavage sites (data not shown). It is likely that similar quantitative differences would be observed in comparing different species, and even different strains of rats, to the results reported herein, obtained using Fischer rats.

For technical reasons, we used concentrations of β-endorphin which are likely considerably higher than that occurring *in vivo.* We infused 2 µl of 1 mM β-endorphin into the striatum in the *in vivo* experiments, and incubated striatal slices with 1 µM β-endorphin. These concentrations are likely to be higher than the overall concentration present in the extracellular fluid of the brain. In vesicles, the concentration of peptides has been estimated to be on the order of 1 mM (34); hence, upon release, the concentration of beta-endorphin would begin to decrease as it diffuses into the extracellular fluid. It is expected that the "concentration" over an entire brain region sampled by microdialysis, for instance, would be much lower than the levels added here (1 mM infusion concentration, 1 uM *in vitro* concentrations). According to one recent study

(35) measuring β-endorphin-like immunoreactivity in microdialysis fractions from the ventral striatum , β-endorphin is present in the fractions at an upper limit of 108 pM. Given estimates of microdialysis recovery of peptides in the range of 1-10%, the actual extracellular concentration of β-endorphin-like immunoreactivity might be presumed to be in the range of 1-10 nM. It is possible that at these lower concentrations, the extent of proteolytic cleavage of β-endorphin will be significantly reduced.

Since immunoblotting experiments have demonstrated that IDE is present in human CSF (30), we explored the possibility that this enzyme is present and functional in rat CSF. Indeed, the observed fragments resulting from β-endorphin-r incubation with rat CSF, namely β-endorphin (1-17), (1-18), (18-31), and (19-31) (Figure 3A), are consistent with the known cleavage sites of β-endorphin-h by purified IDE (25). Furthermore, the attenuation of fragment formation by the metal chelator 1,10-phenanthroline, which inhibits IDE (36), supports the interpretation that IDE is responsible for the observed enzyme activity in rat CSF (Figure 3A). Additional support is provided by our findings that a monoclonal antibody to IDE (9B12) was able to partially immunoprecipitate the enzyme activity from rat CSF (Figure 3B). However, comparing β-endorphin degradation in untreated CSF to that in supernatant following immunoprecipitation of CSF indicates that significant levels of β-endorphin-degrading activity remains. This may be due to insufficient affinity of the monoclonal antibody for IDE to effect complete immunoprecipitation of the enzyme. Studies performed using the same antibody to immunodeplete IDE activity purified from rat muscle showed that the concentration of antibody has sufficient affinity to immunoprecipitate over 80% of the activity, at a concentration of 10 μg/ml (the same concentration used in the present studies) (37). It should be noted that the mass spectra shown in Figure 3B are not quantitative, and the proportion of enzyme activity in the

supernatant to that in CSF may not differ significantly from that observed by Bennett *et al.* (37) with purified IDE. It is also possible that, in addition to IDE, additional enzyme(s) are present in CSF resulting in β-endorphin cleavage at both the Leu^{17}-Phe^{18} and the Phe^{18}-Lys^{19} positions.

The presence of β-endorphin degrading activity in the cerebrospinal fluid of rats has not, to our knowledge, been previously reported. Indeed, in contrast to the present results (Figure 3A), previous studies of β-endorphin-h in human cerebrospinal fluid, using β-endorphin antibodies for radioimmunoassay detection of β-endorphin-like immunoreactivity, indicated that β-endorphin is stable for up to 5 h (38). The apparent stability of β-endorphin in CSF may be an artifact of crossreactivity of the antibody with the resulting β-endorphin fragments, which themselves are stable. It is also possible that the levels of β-endorphin degrading enzymes are higher in rat CSF than in human CSF.

Of the fragments resulting from β-endorphin detected in this study, (1-17) and (1-18) are both predicted to bind to opioid receptors, since they contain the initial enkephalinergic fragment YGGFM (14). Indeed a number of studies have investigated the activity of (1-17) (γ-endorphin) and associated fragments, showing this peptide to have opioidergic, as well as non-opioidergic, activity (Reviewed in 10 and 11). To our knowledge (1-18), which is the primary product observed *in vivo* and in the striatal slice studies, has not been previously confirmed to have opioidergic activity. We determined the binding affinities of the truncation fragments of β-endorphin to the cloned human opioid receptors expressed in CHO cells (Table 1). The relative affinity of full length human β-endorphin at each of the human opioid receptors is very similar to that which has been found previously by other investigators (14). While the affinity of the truncation fragments for the opioid receptors is certainly reduced, compared with full-length β-endorphin, given the somewhat rapid biotransformation observed for β-endorphin *in vivo* (Figure

1), the extracellular biotransformation fragment β-endorphin (1-18) may mediate the pharmacological effects of β-endorphin, following release into the extracellular fluid. β-endorphin (1-18), as well as γ-endorphin and α-endorphin, show no selectivity for MOP-r versus DOP-r, in contrast to β-endorphin, which is approximately two-fold selective for MOP-r. This may result in different pharmacological effects for the truncation fragments of β-endorphin, as MOP-r and DOP-r show important differences in pharmacological effects (39, 40). This difference in relative affinity may also mediate, in part, differences in the effects observed for γ-endorphin versus full-length β-endorphin (10,11). Moreover, pairing of DOP-r activity with MOP-r activity may have subtle but profound effects on overall opioidergic activity (41).

The apparent extracellular localisation of IDE (28, 29) and the cleavage of β-endorphin at the Leu^{17}-Phe^{18} and Phe^{18}-Lys^{19} positions (25), as well as the results discussed here, are consistent with a role of this enzyme in the extracellular biotransformation of β-endorphin. Note, IDE lacks a secretory signal sequence, so the issue of functional *in vivo* extracellular activity of this enzyme has been called into question (27). Moreover, the affinities of the fragments resulting from β-endorphin biotransformation for the opioid receptors indicate that the extracellular biotransformation events described here lead to differentially bioactive peptides which potentially mediate, at least in part, the effects of β-endorphin.

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FIGURE LEGENDS

Figure 1: A representative series of mass spectra resulting from *in vivo* administration of 1.0 nmol β-endorphin via microinfusion, followed by recovery via microdialysis. Following each time duration, the entire 20 minute fraction was collected and subjected to desalting and concentration sample preparation prior to MALDI mass spectrometric analysis. Mass spectra are presented, for clarity, over *m/z* range 800-2200. No intact β-endorphin was generally observed.

Figure 2: Select tandem mass spectra using MALDI-ion trap mass spectrometry, for confirmation of the identity of peptides detected via MALDI-Time-of-Flight mass spectrometry (Figure 1). The fragmentation observed does not provide complete coverage of the peptide sequence; the product ions observed are noted on the spectrum, as well as in the sequence inset.

Figure 3: Results using dissected rat striatum. **A.** Representative mass spectra of β-endorphin processing in the incubation fluid of dissected rat striatum. Following incubating of the striatum for 5 min with phosphate-buffered saline (85 µl), the fluid was removed and aliquoted (20 µl). Following addition of protease inhibitor (bestatin, 500 µM; 1,10-phenanthroline, 1 and 5 mM), β-endorphin (final concentration 5 µM) was added to each aliquot. At various time points, 1 µl was withdrawn for mass spectral analysis. Shown are the mass spectra for the 15-minute time-point. Bestatin clearly inhibits the action of aminopeptidases, whereas 1,10-phenanthroline inhibits the observation of all cleavage events. For clarity, the mass spectra are shown over the *m/z* range of 900 – 3500. **B.** Homogenised rat striatum (str-H) immunoblotting using a polyclonal antibody to insulin degrading enzyme (Chemicon). 30 µg of tissue was loaded onto a 7.5% SDS-PAGE gel, electrotransferred to a PVDF membrane, and immunoblotted. The approximate

molecular weight of the sole band observed above 60 kDa was 110 kDa, which matches that expected from the molecular weight of soluble insulin-degrading enzyme. Additionally, a number of lower molecular weight bands are observed, the identity of which are not known, potentially resulting from proteolytic degradation during the initial homogenization step.

Figure 4: β-endorphin processing *in vitro* in cerebrospinal fluid withdrawn from the rat. Following removal from the cisterna magna, the CSF was immediately frozen, and thawed immediately prior to the experiment. **A.** Representative mass spectra of β-endorphin (5 μM) processing in CSF in the absence and presence of protease inhibitors (bestatin, 100 μM; 1,10-phenanthroline, 1 mM) and β-endorphin (5 μM). At various time-points, 1 μl samples were removed for mass spectral analysis. Mass spectra shown represent the 4.5 hour time-point. Bestatin had no effect on the fragments observed, whereas 1,10-phenanthroline clearly retarded the enzyme activity. **B.** Results of the activity of the immunoprecipitates using a monoclonal antibody to insulin-degrading enzyme. The spectra shown correspond to a cleavage time of 1 hour. The antibody clearly was able to immunoprecipitate the enzymatic activity resulting in β-endorphin cleavage to 1-18, 19-31 and 1-17, 18-31 (bottom spectrum). The immunoprecipitation did not result in complete immunodepletion of the enzymatic activity (middle-spectrum, CSF supernatant).

Figure 5: Sequence of rat β-endorphin with, differences in the sequence in human and porcine β-endorphin noted above (dashed lines indicate no changes). The cleavage sites of β-endorphin by purified insulin-degrading enzyme reported by Safavi *et al*. (10), as well as the β-endorphin fragments observed in the present studies in CSF and the incubation fluid of dissected striatum, are shown for rat β-endorphin.

TABLE 1. K_i Values for the binding of Opioid Peptides to the Human MOP, DOP, and KOP Receptors.

	K_i (nM) ± SEM		
	[^{3}H]DAMGO (MOP)	**[^{3}H]Naltrindole (DOP)**	**[^{3}H]U69,593 (KOP)**
With bestatin/captopril/PMSF			
Beta-Endorphin [h]	4.4 ± 0.3	6.1 ± 1.0	40 ± 1
Beta-Endorphin [r] (human receptor)	1.6 ± 0.1	3.5 ± 0.3	20 ± 1
Beta-Endorphin (1-18) [h,r]	18 ± 1	16 ± 1	300 ± 20
Gamma-Endorphin (1-17) [h,r]	11 ± 1	9.2 ± 0.9	360 ± 30
Alpha-Endorphin (1-16) [h,r]	11 ± 1	8.9 ± 0.3	310 ± 20
Met-Enkephalin [h,r]	2.0 ± 0.3	0.64 ± 0.06	130 ± 20

K_i values for the inhibition of MOP, DOP, and KOP binding by endorphin peptides in the presence and absence of protease inhibitors. Cell membranes prepared from CHO cells that stably expressed either the human MOP, DOP, and KOP receptors were used in the experiments. Membranes were incubated at 25^{0}C in 1 ml of 50 mM Tris-HCl, pH 7.5, along with 12 different concentrations of the peptides in the absence or presence of 30 μM bestatin, 10 μM captopril, and 100 μM PMSF. Data are the mean ± S.E. from at least three separate experiments, performed in triplicate. [h] and [r] refer to human and rat isoforms of the peptide, respectively (see Figure 5).

FIGURE 1

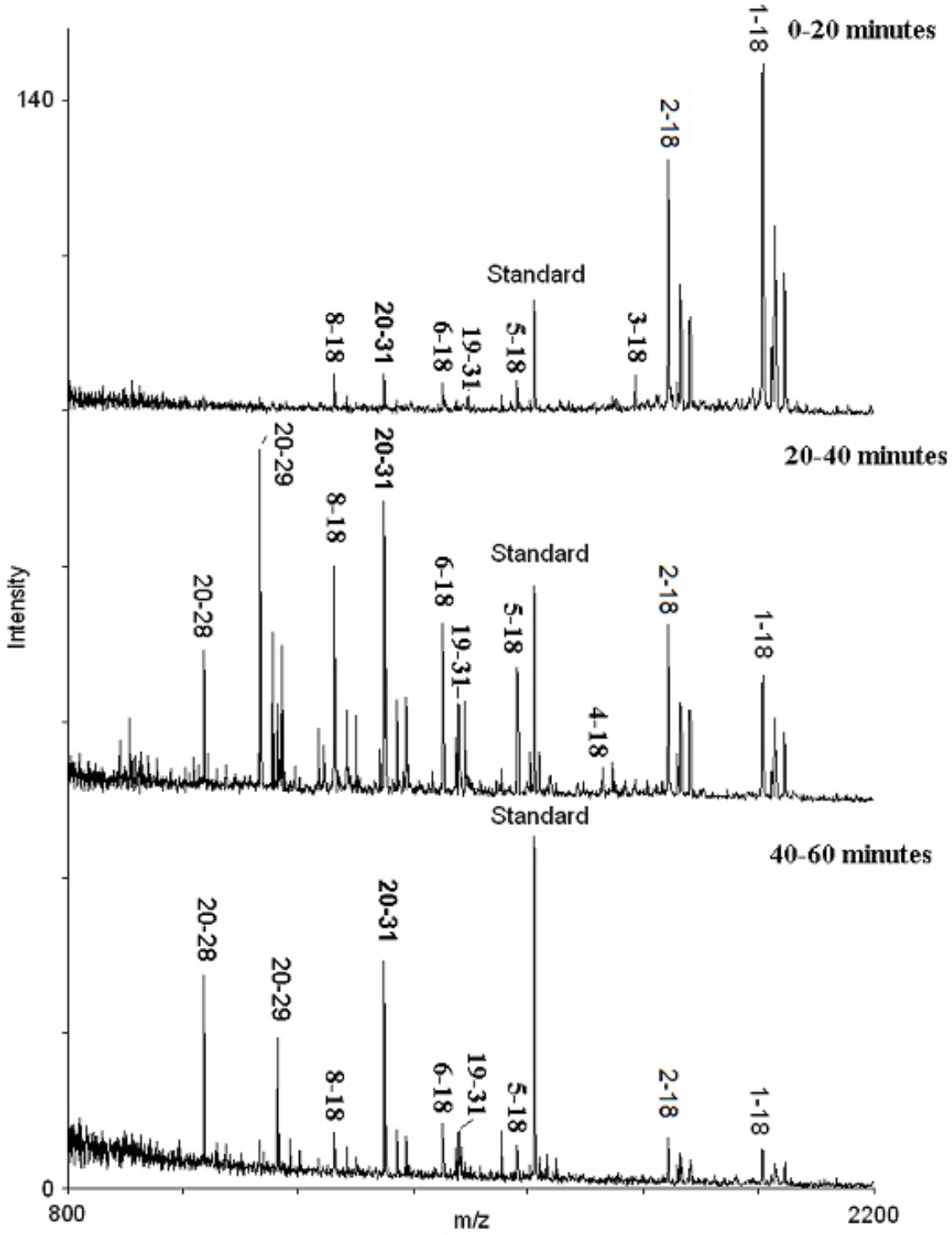

0-20 minutes
1-18
2-18
3-18
Standard
5-18
19-31
6-18
20-31
8-18
20-40 minutes
20-29
20-28
4-18
40-60 minutes
Intensity
140
0
800
m/z
2200

FIGURE 2

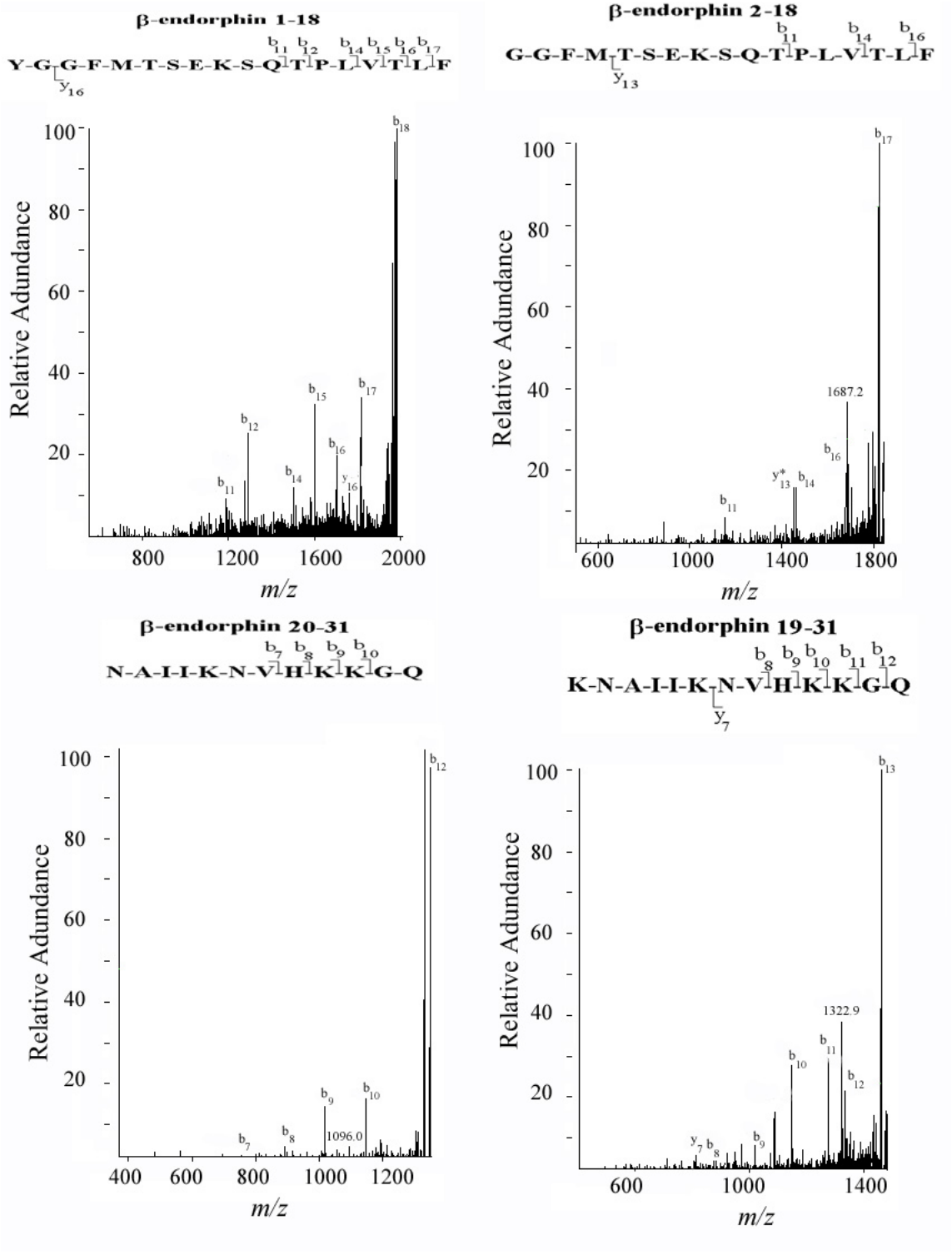

β-endorphin 1-18
Y-G-G-F-M-T-S-E-K-S-Q-T-P-L-V-T-L-F
β-endorphin 2-18
G-G-F-M-T-S-E-K-S-Q-T-P-L-V-T-L-F
β-endorphin 20-31
N-A-I-I-K-N-V-H-K-K-G-Q
β-endorphin 19-31
K-N-A-I-I-K-N-V-H-K-K-G-Q
Relative Adundance
m/z
1687.2
1096.0
1322.9

FIGURE 3

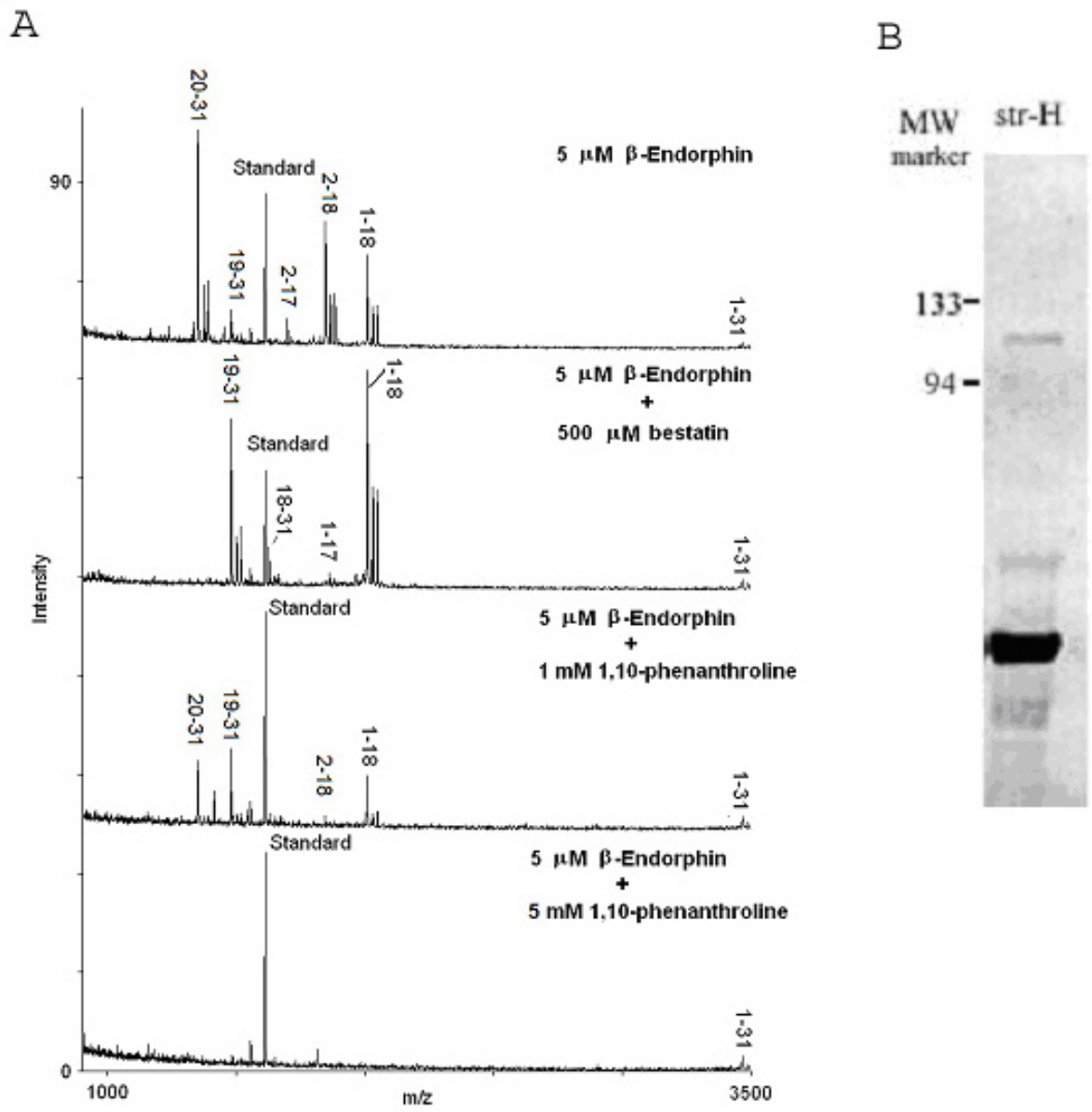

A
B
MW
marker
str-H
133
94
Intensity
90
0
1000
m/z
3500
Standard
20-31
19-31
2-18
2-17
1-18
1-31
5 μM β-Endorphin
5 μM β-Endorphin
+
500 μM bestatin
18-31
1-17
5 μM β-Endorphin
+
1 mM 1,10-phenanthroline
5 μM β-Endorphin
+
5 mM 1,10-phenanthroline

FIGURE 4

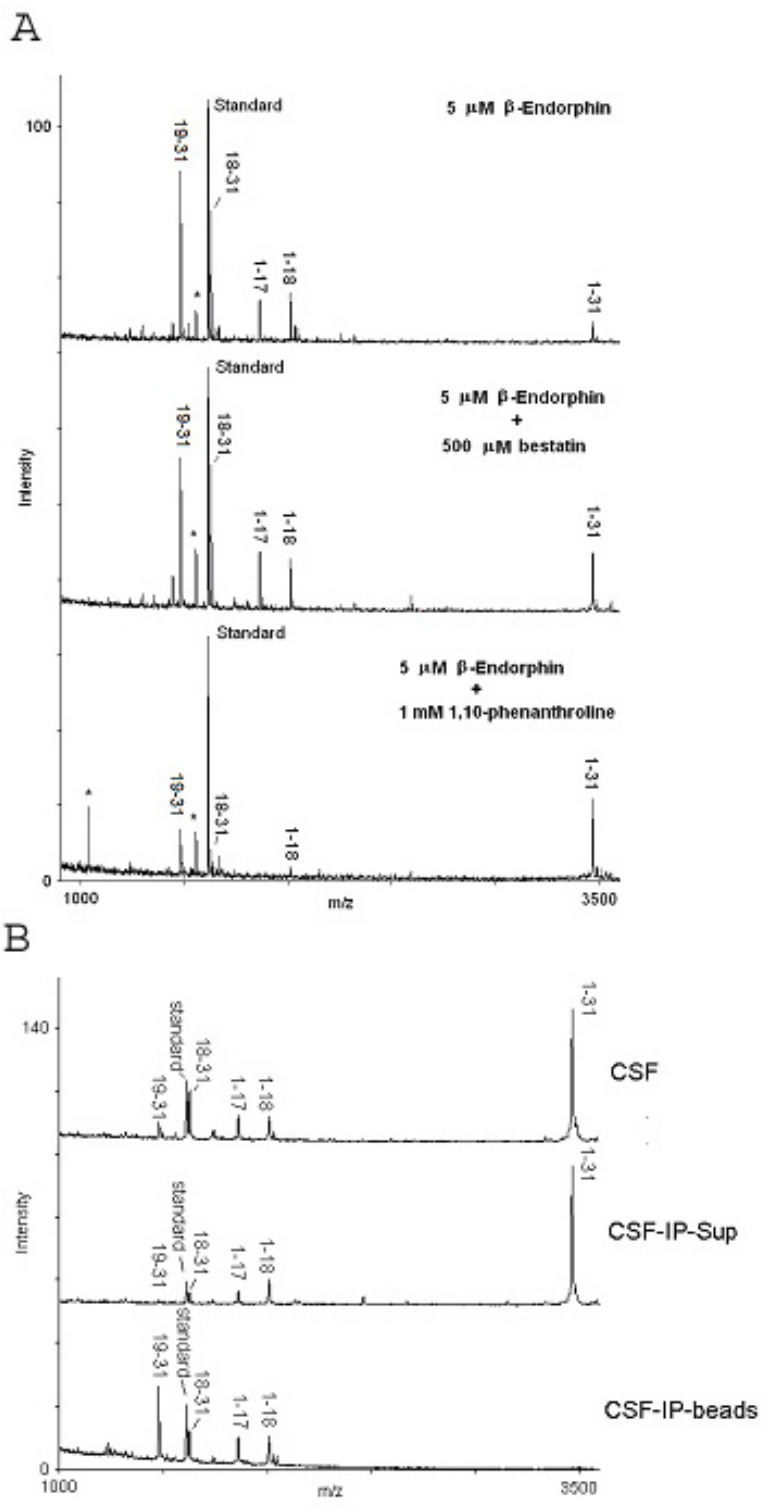
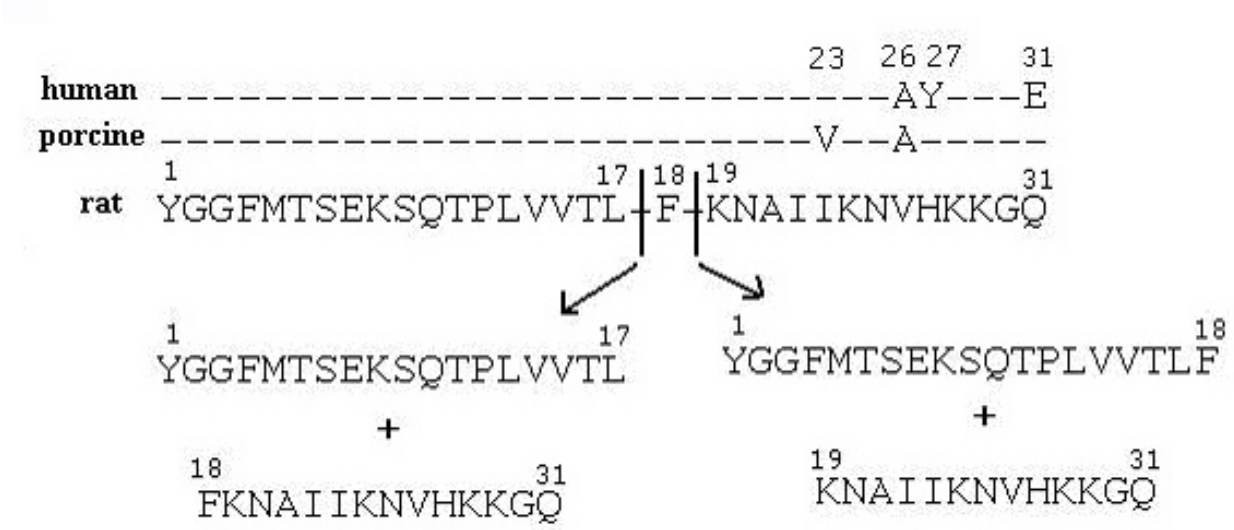

A
Standard
5 µM β-Endorphin
19-31
18-31
1-17
1-18
1-31
5 µM β-Endorphin
+
500 µM bestatin
1 mM 1,10-phenanthroline
Intensity
100
0
1000
m/z
3500
B
standard
CSF
CSF-IP-Sup
CSF-IP-beads
140

FIGURE 5


23 26 27 31
human _____AY___E
porcine _____V__A_____
1 17 18 19 31
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1 17
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+
18 31
FKNAIIKNVHKKGQ
1 18
YGGFMTSEKSQTPLVVTLF
+
19 31
KNAIIKNVHKKGQ