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IDENTIFICATION OF [MET^5]-ENKEPHALIN IN DEVELOPING, ADULT, AND RENEWING TISSUES BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY

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Summary

Extracts of adult corneal epithelium, and developing and adult cerebellum, aorta, and heart, from rats were evaluated for [Met^5]-enkephalin. Samples were prepared by ultrafiltration and solid phase extraction with a C-18 Sep-pak, separated by reversed phase high-performance liquid chromatography, and analyzed by radioimmunoassay (RIA). This qualitative analysis revealed the presence of [Met^5]-enkephalin in all tissues but the adult cerebellum. These results confirm and extend earlier reports that have used RIA or immunohistochemistry with regard to the presence of this opioid peptide in developing and renewing tissues, and indicate that [Met^5]-enkephalin is indeed being recognized by immunological assays.

Key Words: [Met^5]-enkephalin, opioid growth factor, reversed phase HPLC, radioimmunoassay, corneal epithelium, aorta, heart, cerebellum

In addition to neuromodulation (1), the endogenous opioids play a role in the growth of normal, neoplastic, renewing, and healing tissues (2-15). Opioids can function to control growth directly and through an opioid receptor-mediated interaction (e.g., 7-11,14,15) or by way of indirect effects that may not depend upon opioid receptors (5). Although a variety of opioids and opioid receptors have been reported to be involved with growth, the only native opioid found to be directly involved both *in vivo* and *in vitro* has been the pentapeptide, [Met^5]-enkephalin (7-11,14,15). Closely related native peptides, but not synthetic opioids, have shown some growth regulatory properties but to a lesser extent. To distinguish the role of this peptide as a growth factor in neural and nonneural cells and tissues and in prokaryotes and eukaryotes, [Met^5]-enkephalin has been termed opioid growth factor (OGF) (7-11,14,15). OGF is a potent, reversible, species-unspecific, tissue-

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unspecific peptide that is a negative growth regulator. This neuropeptide is autocrine and possibly paracrine produced and secreted, and effective at concentrations consistent with the binding affinity of its receptor. OGF is rapid in biological action, not cytotoxic, and obedient to the intrinsic rhythms of the cell (e.g., circadian rhythm), and acts in a stereospecific and naloxone-reversible fashion consistent with receptor mediation. This peptide is targeted to cell proliferation but also appears to influence cell differentiation, migration, and survival. OGF is tonically active, and interference with peptide-receptor interaction accelerates cell growth by removing inhibitory signals. [Met^5]-enkephalin interacts with μ- and δ-receptors of neuronal cells when serving as a neurotransmitter, but as the OGF it interfaces with an opioid receptor, zeta (ζ), to modulate growth.

The identification of [Met^5]-enkephalin as a growth factor (i.e., OGF) or neurotransmitter has often relied on radioimmunoassay (RIA) or immunohistochemistry. Although powerful, these techniques are limited by the cross-reactivity of the antibodies to compounds other than [Met^5]-enkephalin. The present study was designed to identify the nature of the opioid peptide, putatively reported to be OGF in earlier investigations, in the modulation of cellular growth in a variety of tissues. Our strategy was to use two complementary techniques: reversed phase high-performance liquid chromatography (RP-HPLC) and RIA, to examine the presence of the opioid peptide [Met^5]-enkephalin in the cerebellum, aorta, and heart of developing and adult rats, and the corneal epithelium of adult animals in order to discern whether these tissues do indeed contain this peptide.

Materials and Methods

Animals

Nulliparous female (195-230 g) and male (250-300 g) Sprague-Dawley rats (Charles River Labs, Wilmington, MA) were used in this study and maintained according to conditions described earlier (7-11,14,15).

Animals were mated (1 male to 2 females) and the presence of sperm indicated pregnancy (= day 1 of gestation); all animals were maintained in stainless steel, wire-bottomed cages except where noted. Three days prior to parturition, the pregnant females were separated and placed individually in solid bottomed cages to deliver their pups.

Tissue Collection and Preparation

All rats were anesthetized with sodium pentobarbital, decapitated, and appropriate tissues harvested. To obtain corneal tissue, eyes were enucleated from adult rats and the epithelial layer detached from the underlying stroma by a scalpel. Cerebellum was collected from 6-day old and adult animals, whereas the ascending aorta and ventricles of the heart were isolated from neonatal and adult rats according to earlier reports (8,9,10-13). Immediately following dissection, all tissues were placed in Tris buffered saline (TBS) containing protease inhibitors (7).

The tissues from at least 6 developing rats, and at least 2 adult rats, were used for evaluation. Tissues were homogenized in TBS. Homogenates were spun through a 10,000 MW Centricon filter (Amicon Inc., Beverely, MA) to remove particles and high molecular weight proteins; extracts were frozen and stored at -80^0 C.

HPLC Analysis

Immediately prior to HPLC analysis, tissue extracts were diluted with HPLC grade water and passed through an 0.45 μm filter and lyophilized. One ml of 0.1% trifluoroacetic acid (TFA) was added to each sample and mixed thoroughly. Two additional 1 ml aliquots of 0.1% TFA were used as rinses in the sample vial and also placed onto a C-18 Sep-pak column (Waters, Milford, MA) for a total volume of 3 ml. The Sep-pak was pretreated with 4 ml of methanol, followed by 4 ml of 0.1% TFA. The Sep-pak was washed with an additional 3.5 ml of 0.1% TFA to remove water soluble salts, and further washed with 3 ml of 20% acetonitrile in 0.1% TFA. Enkephalins were eluted with 4 ml of 0.1% TFA in acetonitrile. The eluent was evaporated to dryness in an inert atmosphere, and 500 μl of HPLC mobile phase was added to each sample prior to analysis.

Two HPLC systems were utilized. The first consisted of a Waters Gradient HPLC equipped with a Model 710 WISP and a Model 481 UV detector set at 200 nm. The HPLC column initially was packed with 5 μm Spherisorb ODS-1 (3.9 x 300 mm). The initial mobile phase was a mixture of 88/12 (v/v) 0.1% TFA acetonitrile and the final mobile phase was 40/60 (v/v) 0.1% TFA/acetonitrile, both flowing at 1.5 ml/minute. The gradient was composed of the initial conditions for 10 minutes, followed by a linear gradient to final conditions for 20 minutes. The gradient was held for 10 minutes at final conditions, and returned to the initial conditions in the last 10 minutes. The second HPLC system used equipment as described above, and an isocratic mobile phase consisting of 78/22 (v/v) water/acetonitrile containing 0.1% TFA flowing at 0.65 ml/minute through a 2 μm SuperODS (4 x 150 mm) (Toso Hass, Philadelphia, PA). In the case of the first HPLC system, multiple 50 μl aliquots were injected and peaks occurring at the same retention time as [Met^5]-enkephalin were collected manually for subsequent RIA analysis. Ten μl quantities of the standard materials were injected in order to obtain retention times. Because the second HPLC column had a more limited capacity, multiple 25 μl aliquots were injected and the peaks collected manually. Five μl quantities of the standard material were injected.

Radioimmunoassays

The fractions comprising the peak which corresponded to [Met^5]-enkephalin were collected, pooled, lyophilized, and stored at -80^0 C. Peptides were extracted with cold 0.2 N HCl. All assays for [Met^5]-enkephalin were performed with a RIA kit from Atlantic Antibodies (INCstar; Sillwater, MN). Specificity of the assay was 100% activity for [Met^5]-enkephalin and a cross-reactivity of 2.8% with [Leu^5]-enkephalin, 0.1% with α-endorphin [β-lipotropin-(61-77)], and <0.002% with β-endorphin, α-neoendorphin, substance P, and porcine dynorphin 1-13. Each sample was evaluated in triplicate and two independent assays were performed.

Results

In the first study, the retention time for synthetic [Met^5]-enkephalin was 10.7 minutes (Fig. 1) using a gradient HPLC system; [Met^5]-enkephalin sulfoxide eluted at 4.0 minutes. The entire gradient system required an hour for analysis. Chromatographs obtained from evaluation of adult corneal epithelium extract indicated the presence of a material corresponding to [Met^5]-enkephalin (Fig. 1). Injection of synthetic [Met^5]-enkephalin and adult corneal epithelium extract together produced a peak at 10.7 minutes that was higher than the peak for the adult corneal epithelium (data not shown). Evaluation of the peak for corneal epithelium alone by RIA showed the presence of [Met^5]-enkephalin (Table I).

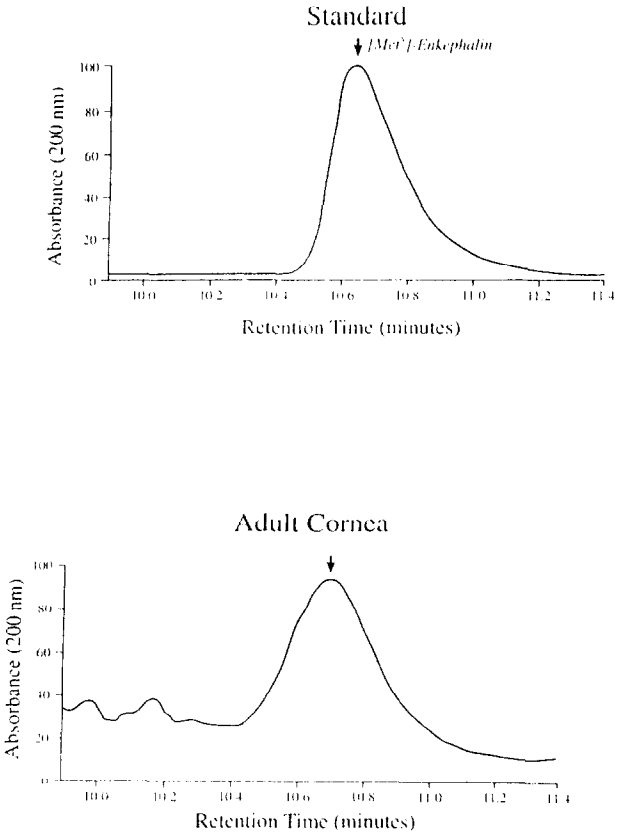

Fig. 1
Chromatographs of HPLC analysis of [Met^5]-enkephalin standard (upper panel) and adult corneal epithelium (lower panel). Arrow indicates an elution time of 10.7 minutes. Absorbances (200 nm) are expressed as a percentage in order to view qualitative differences.

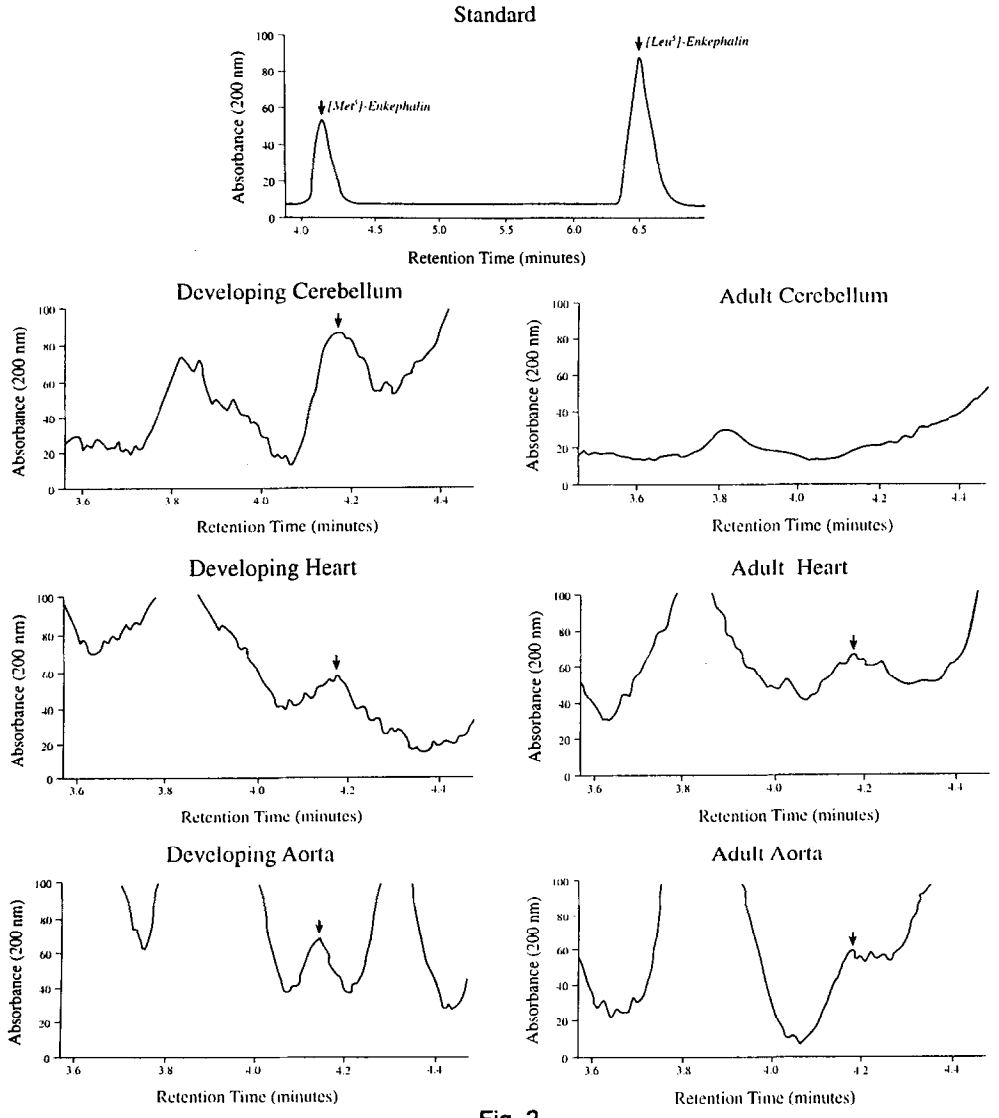

Fig. 2

Chromatographs of HPLC analysis of [Met^5]-enkephalin standard that includes [Leu^5]-enkephalin after isocratic elution; arrows indicate [Met^5]-enkephalin (an elution time of approximately 4.2 minutes) and [Leu^5]-enkephalin (an elution time of 6.5 minutes). Extracts from the developing cerebellum, aorta, and heart, as well as adult aorta and heart, contained a substance that eluted at the same timepoint as [Met^5]-enkephalin (arrow). Extracts from the adult cerebellum exhibited no peak at this timepoint. Absorbances (200 nm) are expressed as a percentage in order to view qualitative differences.

TABLE I

Detection of [Met^5]-enkephalin in Developing and Adult Rat Tissues

Tissue	[Met^5]-enkephalin (pg/ml)
Corneal Epithelium - Adult	850 ± 47
Cerebellum:	
6-day old	8204 ± 69
Adult	Not detected
Aorta:	
Neonate	8937 ± 55
Adult	3250 ± 754
Heart:	
Neonate	7937 ± 47
Adult	571 ± 25

Values represent means ± S.E.

Using an isocratic HPLC system in the second study, a 15 minute analysis time was required. To ensure that no compounds were adsorbed onto the column that could interfere with subsequent evaluations, this column was washed with 100% acetonitrile containing 0.1% TFA at the end of each analysis. The retention time for synthetic [Met^5]-enkephalin was approximately 4.2 minutes. Additionally, we found that [Leu^5]-enkephalin was clearly resolved from the closely related compound [Met^5]-enkephalin, with a retention time of 6.5 minutes recorded for [Leu^5]-enkephalin (Fig. 2). Moreover, [Met^5]-enkephalin sulfoxide eluted at 2.0 minutes. Assessment of the cerebellum, aorta, and heart from developing rats, as well as the aorta and heart from adult animals, revealed the presence of a material corresponding to [Met^5]-enkephalin (Fig. 2). Injection of synthetic [Met^5]-enkephalin and these extracts together produced a peak at 4.2 minutes that was noticeably higher than for peaks of each tissue (data not shown). [Met^5]-enkephalin could not be detected in the adult cerebellum (Fig. 2). Evaluation of the peaks for each tissue alone by RIA indicated the presence of [Met^5]-enkephalin (Table I).

Discussion

The opioid peptide, [Met^5]-enkephalin is known to function as a growth factor in developing neural (cerebellum) and nonneural tissues (aorta and heart), as well as in systems undergoing renewal (corneal epithelium) or cell turnover (aorta) (8-11,14,15). In order to ascertain whether authentic [Met^5]-enkephalin was present in these tissues, and to eliminate problems with cross-reactivity that occur with immunological methods, the present study utilized a series of approaches that

included ultrafiltration, solid-phase extraction, RP-HPLC, and RIA. [Met^5]-enkephalin was detected in all of these tissues. Moreover, the utilization of these orthogonal strategies recorded [Met^5]-enkephalin in the adult aorta and heart, but not in the adult cerebellum. It may be postulated that the presence of [Met^5]-enkephalin in developing and renewing tissues is presumably related to the function of this peptide as a growth factor. In adult heart and aorta, [Met^5]-enkephalin may serve in the capacity of monitoring cell turnover and/or as a neuromodulatory agent associated with innervation.

The present results extend and confirm earlier reports using RIA and/or immunocytochemistry showing that [Met^5]-enkephalin is associated with the developing but not adult cerebellum (11-13), developing aorta (8), developing heart (9), and adult corneal epithelium (14-17). Using a combination of refined procedures for sample preparation that included removal of materials larger than 10,000 MW by ultrafiltration, elimination of water soluble materials by solid phase extraction using a C-18 Sep-pak, and isolation of the peaks of interest by HPLC, followed by RIA, we suggest that the immunoreactivity observed in earlier preparations was related to native [Met^5]-enkephalin. It is interesting to note that McLaughlin (9) did not observe immunocytochemical staining for anti-[Met^5]-enkephalin IgG in the adult rat heart, but that this peptide was found in the adult heart preparations in the present report. This discrepancy may be due to a low abundance of peptide which was undetectable by immunocytochemistry. It may be noted that McLaughlin (9) used immunocytochemistry on fresh sections, and not pretreatment with colchicine, a technique often used to visualize neuropeptides that function in neurotransmission. The present data also are consistent with a study in bovine and canine cornea in which RP-HPLC and RIA were used to detect [Met^5]-enkephalin (17), and the report by Tinsley et al. (16) where [Met^5]-enkephalin was molecularly identified in the bovine cornea by fast atom bombardment-mass spectrometry.

The rationale underlying this study was to determine if the immunological results presented earlier could be substantiated by a variety of different techniques in order to investigate the presence of [Met^5]-enkephalin. This study, however, was not a quantitative analysis of [Met^5]-enkephalin. The efficiency of the initial peptide extraction, as well as the recovery after the chromatography were not corrected by internal standards and thereby limited quantitative analysis. Thus, in future experiments where quantitation is desired, a value for overall peptide recovery on this system would be required.

Acknowledgments

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