JCI The Journal of Clinical Investigation

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J Clin Invest. 1982;69(3):643-650. https://doi.org/10.1172/JCI110491.

Research Article

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Methionine-Enkephalin and Leucine-Enkephalin in Human Sympathoadrenal System and Pheochromocytoma

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ABSTRACT To elucidate the physiological and pathophysiological significance of methionine- and leucine-enkephalin (Met- and Leu-enkephalin, respectively) in human sympathoadrenal system, the contents of these peptides in normal human sympathetic nervous system, adrenal medulla, and pheochromocytomas were determined by specific radioimmunoassays combined with reverse-phase high-performance liquid chromatography. Met-enkephalin-like and Leu-enkephalin-like immunoreactivity (Met-enkephalin-LI and Leu-enkephalin-LI, respectively) were detected by radioimmunoassay in adrenal glands, adrenal medulla, stellate ganglia, sympathetic trunks, and celiac ganglia, and their contents in adrenal medulla were highest. Existence of authentic Met- and Leu-enkephalin was confirmed by reverse-phase high-performance liquid chromatography. Met-enkephalin was ~74% of Met-enkephalin-LI, whereas Leu-enkephalin was ~30% of Leu-enkephalin-LI in human adrenal medulla. The ratio of Met- to Leu-enkephalin was 2.6 in human adrenal medulla, whereas it was higher in sympathetic ganglia or trunks.

In four cases of pheochromocytoma marked difference in Met- and Leu-enkephalin contents was found between medullary and extramedullary tumors. The contents were about three orders higher and the Met- to Leu-enkephalin ratio was lower in medullary than in extramedullary pheochromocytomas, reflecting the tissues where the tumors arose.

These results suggest the physiological roles of Met- and Leu-enkephalin in sympathetic nervous system and adrenal glands and their pathophysiological significances in pheochromocytomas.

INTRODUCTION

Because two related pentapeptides, methionine- and leucine-enkephalin (Met- and Leu-enkephalin, respectively)[1] were identified as endogenous opioid substances (1), evidence has accumulated suggesting that they function as neurotransmitter or neuromodulator in the central nervous system (2). Recent reports have shown that enkephalin-like peptides are widely distributed in peripheral tissues, such as sympathetic ganglia, adrenal glands, and the gut (3–7). Furthermore, multiple molecular forms of enkephalin-like peptides were detected in bovine adrenal gland, either by radioimmunoassay (RIA) or radioreceptor assay in combination with chromatographic methods (8, 9). In addition to the heterogeneity in molecular size, the contents of enkephalin-like peptides have been shown to vary from species to species, especially in the adrenal gland (10).

Recently, Met- and Leu-enkephalin have been demonstrated to exist in human plasma (11, 12), and the sympathoadrenal system, especially adrenal medulla, is supposed to be the source of plasma enkephalins. However, little is known about enkephalin-like peptides in human sympathoadrenal system except the preliminary finding about Met-enkephalin-like material in the adrenal medulla and pheochromocytoma (11, 13, 14).

This study was designed to investigate Met- and Leu-enkephalin in human sympathoadrenal system and pheochromocytoma by sensitive RIA for Met- and Leu-enkephalin in combination with reverse-phase high-performance liquid chromatography (HPLC).

METHODS

RIA. Synthetic Met-, Leu-enkephalin, α-endorphin, and γ-endorphin were generously provided by Daiichi Phar-

Received for publication 8 June 1981 and in revised form 26 October 1981.

[1] *Abbreviations used in this paper*: HPLC, high-performance liquid chromatography; Leu-enkephalin, leucine-enkephalin; Leu-enkephalin-LI, leucine-enkephalin-like immunoreactivity; Met-enkephalin, methionine-enkephalin; Met-enkephalin-LI, methionine-enkephalin-like immunoreactivity; RIA, radioimmunoassay.

maceutical Co., Ltd., Tokyo, Japan. δ-Endorphin (β-lipotropin 61-87) was purchased from Peninsula Laboratories, Inc., San Carlos, Calif. Human β-endorphin was donated by C. H. Li (University of California, San Francisco) and human ACTH by National Pituitary Agency. Dynorphin (1-13) was a gift of A. Goldstein (Stanford University, Calif.).

Antibodies to Met- and Leu-enkephalin (MC3-1210 and LC2-1210, respectively) were raised in New Zealand white rabbits. As antigens, Met- and Leu-enkephalin were conjugated to bovine thyroglobulin (Sigma Chemical Co., St. Louis, Mo.) using the carbodiimide coupling procedure. In brief, 3 mg of Met-enkephalin and 57 mg of bovine thyroglobulin were dissolved in 2 ml distilled water. To this solution was added 30 mg 1-ethyl-3-(3-di-*methyl*-aminopropyl) carbodiimide HCl (Nakarai Chemicals, Co. Ltd., Kyoto, Japan) in 1 ml distilled water. This mixture was adjusted to pH 5.6 with 0.1 N HCl. 3 mg of Leu-enkephalin was conjugated to 56 mg of bovine thyroglobulin in the same way. After stirring for 13 h at room temperature, the mixture was dialyzed against 4 liter of water at 4°C for 24 h. Conjugated Met- and Leu-enkephalin (100–200 μg) were emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) and used for immunizing New Zealand white rabbits by subcutaneous injections at multiple sites in interscapulo-vertebral region. They were boostered every 4 wk and bled 10–14 d after each booster injection.

Met- and Leu-enkephalin were radioiodinated by the chloramine T method of Hunter and Greenwood (15). The labeled Met- and Leu-enkephalin were purified by applying the reaction mixture to a Sep-Pak C_{18} cartridge (Waters Associates, Inc., Milford, Mass.) filled with octadecylsilane-C_{18} (ODS-C_{18}), and eluting the labeled peptides with a solution of 80% methanol in 10 mM ammonium acetate, pH 4.2. ^{125}I-Met- and ^{125}I-Leu-enkephalin were repurified with the same cartridge immediately before use. The specific activity of ^{125}I-Met- and ^{125}I-Leu-enkephalin ranged from 200 to 400 μCi/μg.

The standard buffer for RIA was 0.05 M phosphate buffer (pH = 7.4) containing 0.5% human serum albumin (fraction V, ICN Nutritional Biochemicals, Cleveland, Ohio), 0.1% Triton X-100 and 0.01% merthiolate and used to dissolve all reagents. For RIA, 100 μl of a serial dilution of standard Met-, Leu-enkephalin or sample, 100 μl of a 1:1,000 dilution of MC3-1210 or a 1:2,000 dilution of LC2-1210, 100 μl of ^{125}I-Met- or ^{125}I-Leu-enkephalin (~5,000 cpm), and 200 μl of the standard buffer were mixed. The mixture was then incubated for 24 h at 4°C. Bound and free ligands were separated by adding 1.0 ml of a suspension of dextran-coated charcoal consisting of 300 mg Norit "Extra" (N. V. Norit-Vereeniging, Holland) and 30 mg Dextran T-70 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 100 ml of 0.05 M phosphate buffer, pH 7.4. Samples were assayed routinely in duplicate.

Samples and tissue extraction. Normal tissues were obtained at autopsy within 3 h after death from adult patients without endocrine and neurological disorders. Adrenal medulla was carefully dissected out in cold isotonic saline. Two extramedullary [mediastinal (T.H.) and paraaortic (T.I.)] and two medullary pheochromocytomas (K.S. and M.M.) were also studied. Clinical diagnosis of pheochromocytoma was confirmed by histological examination of extirpated tumors. All tumors were obtained at surgery, immediately cut into slices of ~5 mm^3 in size, and stored at −70°C until extraction. Two slices of each tumor were subjected to subsequent extraction.

Tissue extraction was performed by acidified methanol according to the described procedure (16). In brief, tissues were weighed and homogenized immediately into 10 vol of acidified methanol consisting of equal parts of methanol and 0.1 N HCl. The homogenate was centrifuged at 50,000 *g* for 30 min at 4°C, and the supernatant was stored at −20°C.

The extracted samples were neutralized with 0.1 N NaOH immediately before RIA.

HPLC. The apparatus used for HPLC consisted of a Shimadzu model LC-3A liquid chromatograph equipped with a SIL-IA injector and a variable wavelength UV detector SPD-2A (Shimadzu Corporation, Kyoto, Japan). Reverse-phase HPLC was carried out on a Nucleosil $_7C_{18}$ (Machery-Nagel, Duren, Germany) column (0.4 × 20 cm) isocratically. The extracts were directly applied to the column and eluted with 42% methanol in 10 mM ammonium acetate, pH 4.2, as a solvent. The flow rate was 0.9 ml/min, and the fraction volume was 0.45 ml. The Met- and Leu-enkephalin contents of each fraction were measured by respective RIA. The retention times of synthetic Met- and Leu-enkephalin monitored by ultraviolet absorbance or by RIA were 7.2 and 11.3 min, respectively. Recovery of Met- and Leu-enkephalin standards applied on the column were 94 and 90%, respectively.

RESULTS

RIA. Typical standard curves in the Met- and Leu-enkephalin RIA are depicted in Fig. 1A and B. The cross-reactivities of the antisera (MC3-1210 and LC2-1210) with related peptides were given in Fig. 1A, B, and Table I. Significant inhibition of the binding of ^{125}I-Met- and ^{125}I-Leu-enkephalin to the corresponding antibodies was evident as little as 5 pg of Met- and Leu-enkephalin. The usable range of standard curves is from 5 to 1,000 pg in both RIA. The intra- and interassay coefficients of variation of Met-enkephalin RIA were 2.9 and 11.2%, respectively, while those of Leu-enkephalin RIA were 6.4 and 6.6%, respectively.

On a molar basis the MC3-1210 showed a cross-reactivity of 10.3% with Leu-enkephalin, whereas it had not significant cross-reactivity with β-endorphin, δ-endorphin, γ-endorphin, α-endorphin, and dynorphin (1-13) (Table I).

The LC2-1210 had a cross-reactivity of 12.6% with Met-enkephalin, while it reacted very little with dynorphin (1-13), β-endorphin, δ-endorphin, γ-endorphin, or α-endorphin (Table I).

The dilution curves of extracts from adrenal glands, adrenal medulla, sympathetic ganglia, and pheochromocytomas were parallel with standard curves of Met- and Leu-enkephalin as shown in Fig. 2A and B.

Because the dilution curves of standard Met- and Leu-enkephalin were closely parallel to each other as shown in Fig. 1A and B, Met-enkephalin-like and Leu-enkephalin-like immunoreactivities (Met-enkephalin-LI and Leu-enkephalin-LI, respectively) could be calculated taking cross-reactivities of MC3-1210 and LC2-1210 with the other enkephalin (10.3 and 12.6%, respectively) into account. Met-enkephalin-LI and Leu-enkephalin-LI were determined by resolution of

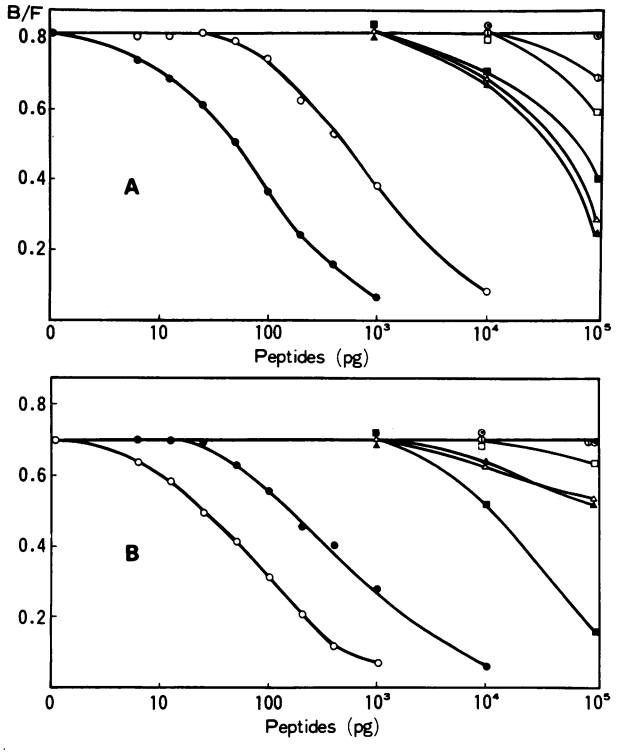

FIGURE 1 Inhibition of bindings of ^{125}I-labeled Met-enkephalin to the Met-enkephalin antiserum (MC3-1210) (A) and ^{125}I-labeled-Leu-enkephalin to the Leu-enkephalin antiserum (LC2-1210) (B) by unlabeled related peptides. Met-enkephalin (— ● —), Leu-enkephalin (— O —), β-endorphin (— ▲ —), δ-endorphin (— Δ —), γ-endorphin (— Φ —), α-endorphin (— □ —), dynorphin (1–13) (— ■ —), and ACTH (— ⊙ —).

the following equations: [Met-E] = Met-enkephalin-LI + 0.103 × Leu-enkephalin-LI; [Leu-E] = Leu-enkephalin-LI + 0.126 × Met-enkephalin-LI. Here, [Met-E] and [Leu-E] were values directly measured by each RIA.

TABLE I
Cross-reactivities of the Met-enkephalin Antiserum (MC3-1210) and the Leu-enkephalin Antiserum (LC2-1210) with Related Peptides

Peptides	Cross-reactivity of MC3-1210	Cross-reactivity of LC2-1210
	%	%
Met-enkephalin	100	12.6
Leu-enkephalin	10.3	100
Dynorphin (1–13)	0.032	0.097
β-endorphin	0.035	0.0032
δ-endorphin	0.028	0.0037
γ-endorphin	0.0031	<0.0015
α-endorphin	0.0098	0.0022
ACTH	<0.0006	<0.0006

Met-enkephalin-LI, Leu-enkephalin-LI, Met-enkephalin, and Leu-enkephalin in normal tissues. The considerable amounts of Met-enkephalin-LI and Leu-enkephalin-LI were detected in human sympathoadrenal system. The contents of Met-enkephalin-LI and Leu-enkephalin-LI in human adrenal glands, adrenal medulla, and sympathetic nervous system are given in Table II. Among them the contents of Met-enkephalin-LI and Leu-enkephalin-LI were highest in adrenal glands especially in adrenal medulla. The content of Met-enkephalin-LI was parallel to that of Leu-enkephalin-LI in each sample.

HPLC patterns of extracts of adrenal glands, adrenal medulla, sympathetic trunks, stellate ganglia, and celiac ganglia revealed two peaks of Met- and Leu-enkephalin immunoreactivity eluting with the same retention times as those of synthetic peptides. The elution profiles of extracts of adrenal glands, adrenal medulla, sympathetic trunks, stellate ganglia, and ce-

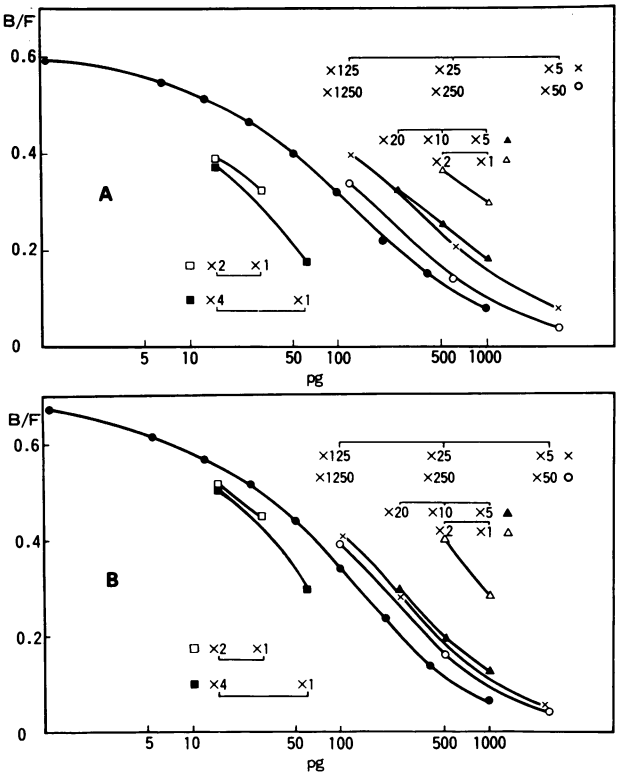

FIGURE 2 Dilution curves of extracts of adrenal glands (— ▲ —) adrenal medulla (— × —), sympathetic trunks (— ■ —), stellate ganglia (— □ —), extramedullary pheochromocytomas (— Δ —), and medullary pheochromocytomas (— O —) in Met-enkephalin RIA (A) and Leu-enkephalin RIA (B). Standard curves of Met-enkephalin or Leu-enkephalin are shown by closed circles (— ● —).

liac ganglia are shown in Fig. 3. Met- and Leu-enkephalin contents of HPLC fractions corresponding to the elution positions of synthetic Met- and Leu-enkephalin were determined (Table II). Although significant amounts of Met- and Leu-enkephalin were present throughout the sympathoadrenal system, the contents of both peptides were highest in the adrenal medulla.

The mean ratios of Met- to Leu-enkephalin in adrenal glands (n = 3) and adrenal medulla (n = 5) were 2.8 and 2.6, respectively. On the other hand, ratios in each sympathetic trunk, stellate ganglia, and celiac ganglia ranged from 4.9 to 11.8.

Met-enkephalin in adrenal glands (n = 3) and adrenal medulla (n = 5) represented ~75.9 and 73.7% of Met-enkephalin-LI, while Leu-enkephalin was ~20.8 and 29.8% of Leu-enkephalin-LI, respectively.

Pheochromocytomas. The amounts of Met-enkephalin-LI and Leu-enkephalin-LI in two extramedullary and two medullary pheochromocytomas are given in Table II. The amounts of Met-enkephalin-LI and Leu-enkephalin-LI in medullary tumors were three orders higher than those in extramedullary tumors. The contents of Met-enkephalin-LI and Leu-enkephalin-LI in extramedullary tumors were compatible with those in sympathetic ganglia, whereas the contents of Met-enkephalin-LI and Leu-enkephalin-LI in medullary tumors were one order higher than those in adrenal medulla.

HPLC patterns of extracts of two extramedullary and two medullary tumors are depicted in Fig. 4. In all extracts there existed two peaks of immunoreactivities with identical retention times to those of synthetic Met- and Leu-enkephalin. The contents of Met- and Leu-enkephalin in pheochromocytomas are shown in Table II. The amounts of Met- and Leu-enkephalin in medullary tumors were one order higher than that of adrenal medulla.

The ratios of Met- to Leu-enkephalin in two medullary tumors were 3.0 and 2.9, while those in two extramedullary tumors were 7.6 and 10.4. The ratios in medullary tumors were similar to those in adrenal glands and adrenal medulla, while the ratios in extramedullary tumors were also comparable to those in sympathetic ganglia.

In medullary pheochromocytoma Met- and Leu-enkephalin were ~88.2% of Met-enkephalin-LI and 46.8% of Leu-enkephalin-LI, respectively, while in extramedullary tumors they were 86.7 and 12.7%, respectively.

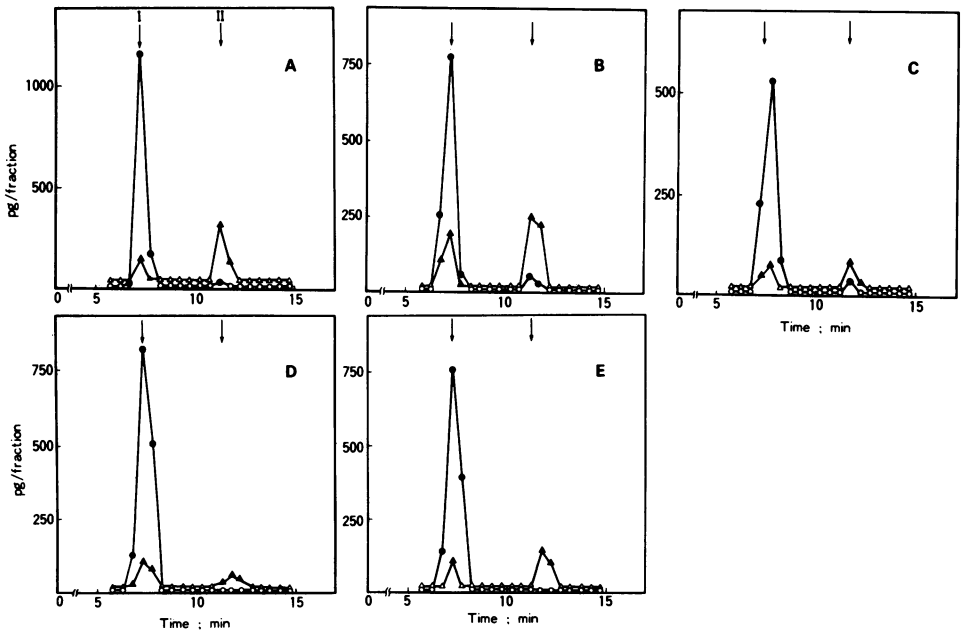

FIGURE 3 High-performance liquid chromatographic profiles on a Nucleosil $_7C_{18}$ column (0.4 × 20 cm) of the extracts of human sympathoadrenal system. A: adrenal gland, B: adrenal medulla, C: sympathetic trunk, D: stellate ganglion, E: celiac ganglion. Fractions of every 30 s were collected and assayed for Met-enkephalin (— ● —) and Leu-enkephalin (— ▲ —) immunoreactivity by respective RIA. Arrows indicate the elution positions of synthetic Met-enkephalin (I) and Leu-enkephalin (II).

TABLE II
Met-Enkephalinlike, Leu-Enkephalinlike Immunoreactivities, and Met-, Leu-Enkephalin in Human Sympathoadrenal System and Pheochromocytoma

	No.	Met-Enkephalin*	Leu-Enkephalin	Ratio of Met- to Leu-enkephalin
		pg/mg	*pg/mg*	
Adrenal gland	3	78.8±16.3‡	28.4±9.0	2.8
	3	(103.8±41.1)	(136.3±58.7)	
Adrenal medulla	5	1471.6±452	558.4±153.6	2.6
	5	(1995.8±554.0)	(1875.2±255.1)	
Sympathetic trunk	1	53.9	8.6	6.3
	2	(28.8±24.9)	(8.8±6.8)	
Stellate ganglion	1	64.8	5.5	11.8
	4	(18.4±4.1)	(4.9±0.9)	
Celiac ganglion	1	10.8	2.2	4.9
	4	(6.5±3.0)	(2.1±0.5)	
Pheochromocytoma				
Extramedullary	Case 1	3.4	0.45	7.6
	T.H.	(3.3)	(2.7)	
	Case 2	44.7	4.3	10.4
	T.I.	(63.5)	(49.3)	
Medullary	Case 1	33300	11100	3.0
	K.S.	(35600)	(24700)	
	Case 2	62200	21500	2.9
	M.M.	(74900)	(44200)	

* Met-enkephalin-LI and Leu-enkephalin-LI are shown in parentheses.
‡ Values represent mean±SE.

DISCUSSION

To elucidate the physiological significance of enkephalins in human sympathoadrenal system, the precise contents of Met- and Leu-enkephalin in these tissues should be determined because marked species difference in contents of enkephalin-like substances in the sympathoadrenal system has been reported (10). In the RIA for enkephalins, however, cross-reactivities of other enkephalin and related peptides are inevitable due to the resemblance in structure. In our RIA for Met- and Leu-enkephalin, there were cross-reactivities of ~10% to each other. Taking these cross-reactivities into account, we calculated Met- and Leu-enkephalin-LI by resolving the equations based on the observations that dilution curves of standard Met- and Leu-enkephalin, as well as tissue extracts, were parallel, and that cross-reactivities of other known related peptides were minimal. A similar analysis was reported by Gros et al. (17).

Because it is difficult to obtain fresh samples from normal human tissues without apparent postmortem proteolysis, sampling conditions are also critical to measure the contents of enkephalins in sympathoadrenal system. In this study specimens at autopsy within 3 h after death were chosen as possible fresh samples.

Our study has demonstrated that both Met- and Leu-enkephalin-LI exist in human sympathoadrenal system. The content of Met-enkephalin-LI in human adrenal medulla was comparable to that reported preliminarily by Clement-Jones et al. (11) and about four times lower than that of bovine adrenal medulla (10). Such difference may be explained by species difference. It may be caused by different RIA systems because multiple molecular forms of enkephalin-like peptides cross-react differently with different antisera.

The existence of Leu-enkephalin-LI was previously shown in bovine adrenal glands (8, 10) but not in human adrenal glands (14) when studied by an immunohistochemical method. We have clearly demonstrated that Leu-enkephalin-LI exists in an amount comparable to that of Met-enkephalin-LI in human

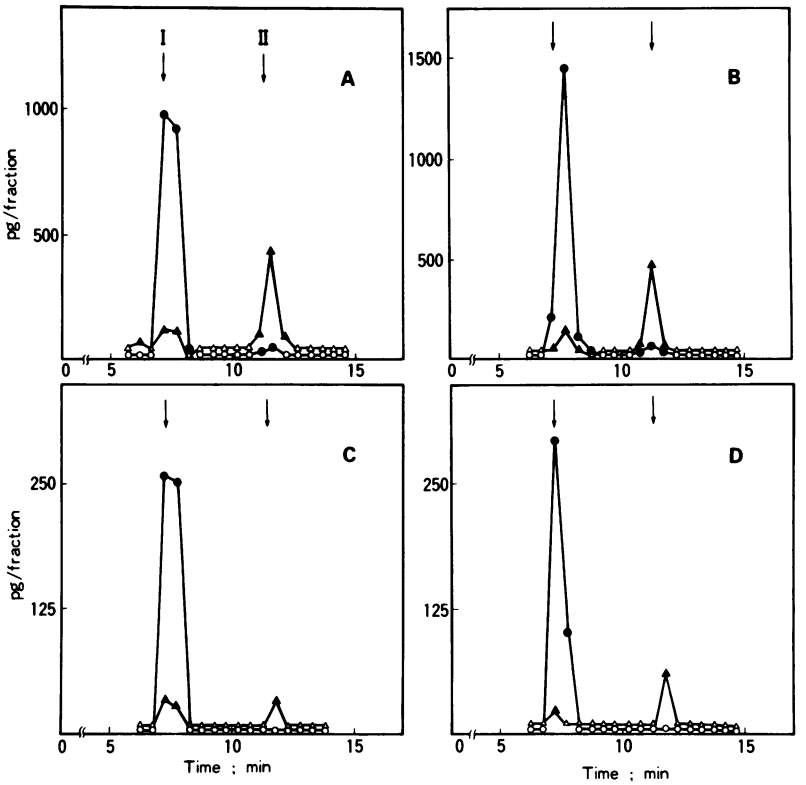

FIGURE 4 HPLC profiles on a Nucleosil $_7C_{18}$ column (0.4 × 20 cm) of the extracts of two medullary pheochromocytomas (A: case 1, B: case 2) and two extramedullary pheochromocytomas (C: case 1, D: case 2). Fractions of every 30 s were collected and assayed for Met-enkephalin (— ● —) and Leu-enkephalin (— ▲ —) immunoreactivity by respective RIA. Arrows indicate the elution positions of synthetic Met-enkephalin (I) and Leu-enkephalin (II).

adrenal glands. In addition, both Met- and Leu-enkephalin-LI were shown to exist in stellate and celiac ganglia and sympathetic trunks. Their contents were lower than those of reported (6) corresponding tissues in Wistar Kyoto rats. The finding that Met- and Leu-enkephalin contents were parallel in each specimen are consistent with the recent finding of Lewis et al. (9) that there exists a common precursor of Met- and Leu-enkephalin with a mol wt of ~50,000 in bovine adrenal gland.

To demonstrate the existence of Met- and Leu-enkephalin more clearly, we used HPLC analysis. The analysis showed that Met-enkephalin measured after the chromatography was ~74% of Met-enkephalin-LI and that Leu-enkephalin was ~30% of Leu-enkephalin-LI. This discrepancy could be accounted for by different cross-reactivities of possible enkephalin precursors with two antisera. Similar observation that high molecular weight forms occupied ~70% of enkephalin-like immunoreactivity in bovine adrenal gland was made by Hexum et al. (10). Recently we have detected dynorphin-like immunoreactivity by RIA for dynorphin (1–13), one of Leu-enkephalin-containing peptides, in human sympathoadrenal system (18). The result indicates the existence of high molecular weight forms of Leu-enkephalin.

The ratios of Met- to Leu-enkephalin in human adrenal glands and adrenal medulla were 2 to 3, whereas those in bovine adrenal medulla were reported to range from 5 to 10 (8, 10). This difference suggests the possible different posttranslational processing of the enkephalin precursor between the species. The ratio of Met- to Leu-enkephalin in human sympathetic ganglia was higher than that of adrenal glands. This result again suggests that the processing varies in different regions of sympathoadrenal system. These differences in absolute and relative amounts of Met- and Leu-enkephalin between sympathetic nervous system and adrenal medulla suggest their different physiological significance in these tissues.

Recently Met- and Leu-enkephalin have been demonstrated to exist in canine and human plasma (11, 12), and shown to be released into blood stream in response to the adrenomedullary stimulation (12,

19). At present, adrenal glands are considered to be the source of plasma enkephalins. High contents of Met- and Leu-enkephalin in human adrenal medulla elucidated in this study are consistent with this hypothesis and suggest possible role of adrenal enkephalins as hormones.

Previous studies have shown that Met-enkephalin-LI is present in pheochromocytoma in a large amount (13, 20). On the other hand Leu-enkephalin-LI has not been demonstrated to exist by either RIA (20) or immunohistochemistry (14). We have clearly demonstrated the presence of Leu-enkephalin-LI as well as Met-enkephalin-LI in pheochromocytoma and also identified the occurence of authentic Met- and Leu-enkephalin by HPLC. Recently we have also demonstrated the presence of dynorphin in pheochromocytoma (21). These results indicate that pheochromocytomas elaborate Met- and Leu-enkephalin as well as their possible precursors.

The most interesting finding in our study is a marked difference in enkephalin contents between medullary and extramedullary pheochromocytomas. The concentrations of Met- and Leu-enkephalin in medullary tumors were about three orders higher than those of Met- and Leu-enkephalin in extramedullary tumors. The difference might reflect the origin of tumors because similar difference in contents has been shown between adrenal medulla and sympathetic ganglia.

The ratios of Met- to Leu-enkephalin in two medullary pheochromocytomas were 2.9 and 3.0, which are similar to the ratios in the normal adrenal gland and adrenal medulla. In extramedullary tumors the ratios were 7.6 and 10.4, which are similar to those in sympathetic ganglia. Therefore, Met- to Leu-enkephalin ratios in medullary and extramedullary pheochromocytomas might also reflect the ratios in tissues where the tumors are derived.

The physiological roles of Met- and Leu-enkephalin in human sympathoadrenal system and the pathophysiological significance of these peptides in pheochromocytoma must await further clarification.

ACKNOWLEDGMENTS

We are indebted to Prof. A. Goldstein, Stanford University, Calif., for the generous supply of dynorphin (1–13); to Professor C. H. Li, University of California, San Francisco, for the generous supply of human β-endorphin; to the Daiichi Pharmaceutical Co., Ltd. for the gift of Met-, Leu-enkephalin, γ-endorphin, and α-endorphin; to Drs. Y. Yoshimasa, S. Yamada, and S. Hamada, Tenri Hospital, Nara, Japan, for the generous supply of tumor samples; to Dr. S. Terabe, Department of Technology, Kyoto University, Kyoto, Japan, for valuable technical advice; to Dr. S. Oki and Dr. I. Tanaka for valuable discussions. We also thank Miss Y. Mitsuda and Miss K. Horii for the secretarial assistance.

This work was supported in part by research grants from the Japanese Ministry of Health and Welfare and from the Ministry of Education, Science, and Culture.

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