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DE NOVO BIOSYNTHESIS OF ENKEPHALINS AND THEIR HOMOLOGUES IN THE HUMAN PLACENTA

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SUMMARY

Fresh trophoblastic preparations of two human placentae delivered at term were pulse labelled for 30, 120 and 240 min with tritiated L-tyrosine. After deproteinizing and defatting, the peptide extracts were first concentrated through reversible hydrophobic binding on octadecasilyl-silica particles, prior to further resolution by repetetive high-performance liquid chromatography. Four peptides were isolated and purified to radioactive homogeneity, namely Met-enkephalin, Leu-enkephalin, [Arg^6]-Leu-enkephalin, and [Arg^6,Arg^7]-Leu-enkephalin. Their presence and identity were further confirmed by substractive Edman degradation and by radioimmunoassay. No detectable amounts of radioactive Dynorphin could be trapped, however. Under the incubation conditions used, reference tritiated Leu-enkephalin had a biological half-life of circa 9.5 min.

INTRODUCTION

Leu-Enk[1] was one of the first opioid-active peptides isolated and chemically identified by Hughes, et al.(1) from porcine pituitary glands. Together with Met-Enk, this pentapeptide pair has later been found in varying ratios also from extracerebral tissues, for example the myenteric plexus (2) and the adrenal medulla (3-6). We are interested not only in the biosynthetic origin of Leu-Enk, but also in its presumed function as a neurotransmitter. The human placenta appeals to us a potentially relevant biological system to carry out the above type of studies. First, it is capable of synthesizing the protein hormones hCG and hCS that are structurally related to the gonadotrophins and GH of the pituitary. Second, several authors have recently shown (7,8) that the human placenta is also able to synthesize pro-opiocortin, the

[1]Abbreviations: Met-Enk, methionine-enkephalin; Leu-Enk, leucine-enkephalin; [Arg^6,Arg^7]-Leu-Enk, H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-OH; ACN, acetonitrile; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography.

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common precursor of β-LPH and ACTH, originally isolated from the pituitary (9-11). Stimulated by these structural and biochemical analogies, we have looked into the possible presence and *in situ* formation of Leu-enkephalin and its putative precursor in the human placenta. Using the pulse-labelling approach we have found that placental trophoblast slices have indeed the capacity of incorporating radioactive amino acids into Leu-enkephalin and other related peptides, details of which we report in the following.

EXPERIMENTAL PROCEDURE

Materials. L-(2,3,5,6-^{3}H)Tyrosine (104 Ci/mmol) was purchased from Amersham Corp. Tyrosyl-ring tritiated Leu-enkephalin (34 Ci/mmol) was from New England Nuclear Corp. Reference Leu-Enk and Met-Enk were from U.S. Biochem. Corp., Dynorphin from Peninsula Laboratories, while (Arg^{6})Leu-Enk, (Arg^{6},Arg^{7})-Leu-Enk and (Arg^{6},Lys^{7})-Leu-Enk were synthesized by the solid phase method by Dr. St. Pierre in the Pharmacology department. Rabbit antiserum against Leu-enkephalin was obtained from Miles-Yeda Ltd., (Tyrosyl-^{125}I)-Leu-Enk was from New England Nuclear Corp. Reference PTH-amino acids and reagents for peptide sequencing were from Pierce Chemical Co. Acetonitrile (HPLC grade) was purchased from Fisher Scientific Co. HPLC-quality water was prepared in our laboratory by double distillation in an all-glass apparatus and final removal of organic contaminants by gravity filtration over the contents of 2 Sep-Pak C_{18} cartridges (Waters Assoc.). Trifluoroacetic acid (Aldrich Chemicals) was distilled prior to use.

High-performance liquid chromatography. Our HPLC system consisted of: a Varian model 5020 chromatograph, a Valco loop injector, a Whatman Partisil ODS-3 (4.6 x 250 mm, 10μ particles) column, a Hitachi-Altex variable wavelenght detector connected in series with a Gilson model 3301 fluorescence monitor, a LKB fraction collector, and a potentiometric recorder. Except for the measurement of PTH-amino acids (260 nm), the UV-detector was kept at 215 nm Parameters employed for the actual chromatographic separations are indicated in the legends and the text.

Measurement of radioactivities. For samples containing tritium, aliquots of the collected fractions were mixed with Aquasol (New England Nuclear) in polyethylene minivials and counted with a Beckman LS 8000 instrument. For the radioimmunoassay, radioactivity was counted with a Beckman 9000 gamma counter.

Pulse-labelling experiments. Two fresh human placentae, obtained at term, were trimmed free of cords and adhering membranes. The trophoblastic shells were collected, cut into small pieces, and washed extensively with ice-cold isotonic KCl solution. The pieces (wet weight 81 g) were filtered over cheese cloth, suspended in KRBG buffer of pH 7.0 (containing 0.154 M NaCl, 6.2 mM KCl, 1.5 mM KH_2PO_4, 1.2 mM $MgSO_4$, 32 mM $NaHCO_3$, 15 mM glucose, 2.2 mM $CaCl_2$, 0.1% BSA and 120 units pennicilin-G/ml buffer). The pieces were minced further into circa 2mm thin slices, filtered over cheese cloth, and distributed in 3 incubation flasks: A (50 g tissue in 75 ml of buffer), B and C (10 g tissue in 15 ml of buffer each). Flask A was used for the actual pulse labelling experiments, flask B was boiled for 10 min and served as a control, while flask C was used to monitor the metabolism of reference Leu-enkephalin by the placental slices. After the addition of a mixture of 19 amino acids, except tyrosine, at a concentration of 0.1 mM each, the flasks were gently shaken in a Dubnoff waterbath at 37° under 5% CO_2/95% O_2 atmosphere. After

20 min of this preincubation were added to flask A and B, respectively 174 µCi and 6 µCi of (^{3}H)Tyrosine, while 1 µCi of (^{3}H)-Leu-enkephalin was added to flask C. The incubations were then continued for 30, 120 and 240 min. They were terminated by adding an equal volume of Bennett's extraction medium (12), and 1 mM of non-labelled tyrosine.

Extractions. The placental suspensions were then homogenized at 4° in a Waring blender and centrifuged for 10 min at 10 000 x g.To the supernatant was added a solution of 50% TCA until a final concentration of 10% TCA was obtained. After settling in the cold room for 3 hours, the precipitate was removed by centrifugation (10 000 x g, 20 min). The supernatant was then carefully defatted by repeated extractions with small volumes of ether in the cold until a clear solution was obtained.

Purification and analyses of peptides. The deproteinized and defatted peptide extracts were first concentrated on, then eluted from 2 Sep-Pak C_{18} cartridges connected in series, according to the microanalytical procedure described previously (6). The peptides present in the 80% ACN/0.1% TFA eluates were then either lyophilized, or vacuum-dried, prior to their resolution and purification by repetetive gradient-, followed by isocratic HPLC. Small aliquots of the collected HPLC fractions were removed and counted in Aquasol. The remainder of the fractions were dried in vacuum and stored at -20° for later use and for radioimmunoassay.

Peptide sequencing. Automated sequencing of 200 µg of Dynorphin was performed using a modified Illitron model 9001 sequenator with a 0.33 M Quadrol program (13). The sequenator was prerun for 3 cycles with 4 mg of polybrene and 100 nmoles of glycyl-glycine (14). Samples were converted manually to the PTH-amino acids by heating in 0.25 M of TFA at 60° for 10 min. They were subsequently separated by HPLC using a Waters model 204 instrument, equipped with a Waters solvent programmer and a Dupont cyanopropylsilane column (15). Smaller peptides were sequenced manually by substractive Edman degradation(16).

Radioimmunoassay. A stock solution of the lyophilized rabbit anti-Leu-Enk-BSA serum (Miles-Yeda) was made by dissolving it in 5 ml of Tris-HCl buffer (50 mM, pH 7.5 containing 2 mM EDTA). The working solution of the antiserum was then prepared by 10 fold dilution of this stock solution with the same buffer. Simultaneously, from a stock solution of Leu-enkephalin (1 µg/ml) a series of standards was prepared ranging from 0.37 ng/ml to 50 ng/ml in Tris buffer. (^{125}I)-Leu-Enk (911 µCi/µg) was diluted with Tris buffer to give a final concentration of 1.6 x 10^5 cpm/ml. The lyophilized fractions collected from gradient HPLC and containing the unknown peptides were then assayed for Leu-enkephalin activity as follows. They were dissolved in 600 µL buffer. 100 µL of it was mixed with 100 µL of the diluted anti-serum and 100 µL of the radio-iodinated Leu-Enk. A parallel assay was conducted using the Leu-Enk standards. Zero controls and blanks were prepared in triplicate with 5 mM phosphate buffer, pH 7.5 substituting for the deleted components. The test tubes were incubated at 4° for 20 hours. The incubations were terminated by the addition of 0.5 ml of phosphate buffer. The content of each assay tube was then immediately vacuum-filtered using Whatman GF/B paper. Each filter was washed with 7.5 ml of phosphate buffer, dried and counted in the Beckman gamma counter. The standard curve is made by plotting on semi-logarithmic paper the % bound versus the log-dose of standard. The % bound of each peptide fraction was then calculated according to Weissman (17). Each peptide was assayed in duplicate.

RESULTS AND DISCUSSION

We have reported earlier (6) that more than 94% of Leu-Enk was retained by a single Sep-Pak C_{18} cartridge when applied in 0.1% TFA solution in the absence of any organic modifier. Consequently, we have adopted the same micro-

TABLE I. DEGRADATION OF (^{3}H)LEUCINE ENKEPHALIN BY HUMAN PLACENTAL SLICES

Incubation time (min)	Radioactivity in HPLC fraction (cpm)	Radioactivity in the total incubation extract (cpm)
30	629	20977
60	125	4168
120	47	1590

(^{3}H)Leu-enkephalin (320 000 cpm) was incubated with fresh human placental slices for 30, 60 and 120 min. After removal of proteins by TCA precipitation and defatting, the three peptide extracts were dissolved in 0.1% TFA and individually analyzed by repetetive HPLC. Reference nonlabelled Leu-enkephaline (1 µg) was co-injected into the column as an absorbance marker.

analytical purification procedure for the present study. It should be noted however, that (^{3}H)tyrosine was also retained by the C_{18} cartridge to the extent of some 10%. Table I shows the "decay" of (^{3}H)Leu-Enk used as a substrate after incubation with the placental slices. From its disappearance curve a biological half-life of circa 9.5 min can be calculated. It is obvious that this pentapeptide undergoes active catabolism and any quantitative statement concerning its concentration in tissues must take this fact into account. In Fig. 1, we have compared the radiochromatograms obtained after the first gradient HPLC of the 120 and 240 min pulse incubations. No significant differences were observed between the 30 and 120 min chromatograms. It can be seen that the peak due to (^{3}H)tyrosine is twice as prominent after the 120 min pulse than after the 240 min pulse. The reason being that after 2 hours, there is less incorporation of radioactive tyrosine. Therefore, relatively more of it is present in the peptide extract, and consequently, more of it is retained by the C_{18} cartridge. The pattern is reversed when we look at the incorporation of radioactivity into peptides, for instance at the peak co-eluting with (Arg^6,Arg^7)-Leu-Enk (fraction 23). Here the radioactivity is more pronounced after 4 hours, than after 2 hours of pulse. Fig. 2 represents an actual gradient HPLC absorbance chromatogram obtained from a 4 hour incubation, superimposed on the histogram of the radioimmuno-

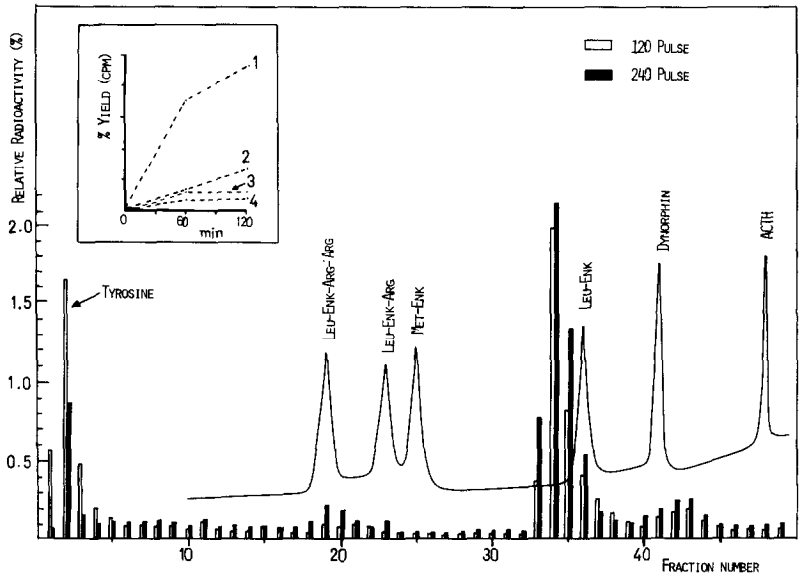

Fig.1. RADIOCHROMATOGRAM OF THE 120 AND 240 MIN PULSE INCUBATION EXTRACTS. 50 g of placental slices were pulsed with 174 µCi of (^{3}H)tyrosine for 120 and 240 min. After treatment with two C_{18} cartridges, the concentrated extracts were injected into the ODS-3 column. A two-phase linear gradient was applied from 15-21.5% ACN for the first 35 min, then to 54% ACN for the next 30 min; 0.1% of TFA was present throughout. Solvent A was 15% ACN, solvent B 80% ACN. Flowrate: 1.5 ml/min. Fractions of 1 ml/tube were collected and 100 µL aliquots counted. Insert: time curve of the 120 min pulse showing the formation of (Arg^6,Arg^7)-Leu-Enk (1), (Arg^6)-Leu-Enk (2), Leu-Enk (3), and Met-Enk (4).

assay of the corresponding fractions. Fraction 36, containing the bulk of the material co-eluting with reference Leu-Enk has the highest immunoreactivity. Fraction 25, co-eluting with Met-Enk contains about 20% of the reactivity of the Leu-Enk fraction. Fraction 43 merits particular attention as it shows not only immunoreactivity, but a significant incorporation of radioactive tyrosine as well (cf. also Fig. 1). This as yet unidentified peptide elutes in our HPLC system just after Dynorphin, but before $ACTH_{1-39}$, and may well be a larger peptide containing the -Tyr-Gly-Gly-Phe-Leu- sequence. At present we are accumulating more of this material to enable us to determine its chemical identity. Most of the peaks eluting before the (Arg^6,Arg^7)-Leu-Enk peak also show, interestingly, native fluorescence, but none of them were immunoreactive against the Leu-Enk antiserum.

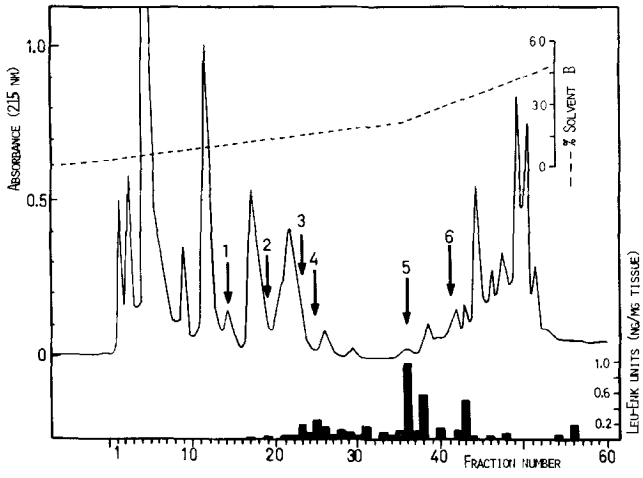

Fig.2. ABSORBANCE AND RIA CHROMATOGRAM OF THE 240 MIN INCUBATION EXTRACT OBTAINED BY GRADIENT HPLC. The chromatographic parameters are as described in Fig.1. Aliquots of the collected fractions were assayed in duplicate for immunoreactivity against the rabbit Leu-enkephalin antiserum from Miles-Yeda using (^{125}I)Leu-Enk as the radioactive ligand. 1 = (Arg^6,Lys^7)-Leu-Enk; 2 = (Arg^6,Arg^7)-Leu-Enk; 3 = (Arg^6)-Leu-Enk; 4 = Met-Enk; 5 = Leu-Enk; 6 = Dynorphin.

The fractions containing the immunoreactive peaks co-eluting with reference enkephalins were collected, concentrated, and subjected to a second and third gradient HPLC using different elution programs. Final purifications were performed by repetetive isocratic HPLC. The peaks were judged homogeneous when a Gaussian distribution of radioactivity was obtained (see Table II). In the case of Met-Enk and Leu-Enk, radioactive purity was proved by their specific activity which remained constant after 2 isocratic HPLC purifications (see Table III). Each of the thus purified peptides were then sequenced by the substractive Edman procedure, either manually, or in the case of Dynorphin, with an automated sequenator. The results are shown in Fig. 3. It is evident that all the radioactivity of the enkephalins is incorporated exclusively at the tyrosine amino terminus. Although the fractions co-eluting with Dynorphin contained initially a low amount of radioactivity (they lack, however, immunoreactivity), after going through several HPLC purifications and the complete sequencing cycles, none of the PTH-amino acids cleaved were found to be radioactive. Furthermore, after trypsinization of the "Dynorphin" peak, the hexa-

TABLE II. FINAL PURIFICATION OF RADIOACTIVE ARGININE HOMOLOGS OF LEUCINE-ENKEPHALIN BY ISOCRATIC HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Fraction*	(Arg^6)-Leu-Enk (R_t: 5.5 min**)		(Arg^6,Arg^7)-Leu-Enk (R_t: 4.4 min**)		(Arg^6,Lys^7)-Leu-Enk (R_t: 3.7 min**)	
	Sample	Control	Sample	Control	Sample	Control
	cpm		cpm		cpm	
1	24	19	22	20	31	32
2	68	22	28	18	34	31
3	89	36	45	19	32	28
4	147	36	404	17	36	32
5	93	23	44	21	24	26
6	62	21	32	18	27	31
7	34	26	67	17	30	28

*Fractions 1 to 3, and 5 to 7 refer to the tubes, preceeding and following, respectively, the single absorbtion peak which was collected as Fraction 4.
**25% ACN/0.1% TFA; ODS-3 column; 1.0 ml/min flow rate; 1.0 ml/tube collected. The fractions co-eluting with reference Met-Enk (R_t: 7.4 min) and Leu-Enk (R_t: 10.8 min) originating from the boiled control samples had only background radioactivity countings. The fraction, co-eluting with (Arg^6,Lys^7)-Leu-Enk, part of α-Neoendorphin, was also devoid of radioactivity.

peptide (Arg^6)-Leu-Enk formed, was also devoid of any radioactivity (see insert, Fig. 3C). It seems therefore, that $Dynorphin_{1-13}$ is neither biosynthesized in the adrenal medulla (6), nor in the human placenta. On the other hand, this study clearly establishes that *de novo* biosynthesis of Enkephalins in the human placenta indeed occurs.

TABLE III. FINAL PURIFICATION OF MET- AND LEUCINE-ENKEPHALIN TO CONSTANT SPECIFIC RADIOACTIVITY BY ISOCRATIC HPLC

Run No.	Met-Enkephalin			Leu-Enkephalin		
	cpm	area	ratio	cpm	area	ratio
1	397	17.08	23.24	1024	10.94	93.60
2	110	12.82	8.58	291	7.99	36.42
3	79	9.05	8.66	302	8.27	36.51

The peaks co-eluting with 2 μg of reference Met-Enk and Leu-Enk were pre-purified by 3 successive gradient HPLC runs, followed by isocratic HPLC (ODS-3 column; 25% ACN/0.1% TFA; 1.0 ml/min). After the third isocratic run, the total fraction containing the enkephalin peaks was vacuum-dried, then counted in Aquasol. The UV-absorbing peak areas were quantitated by triangulation.

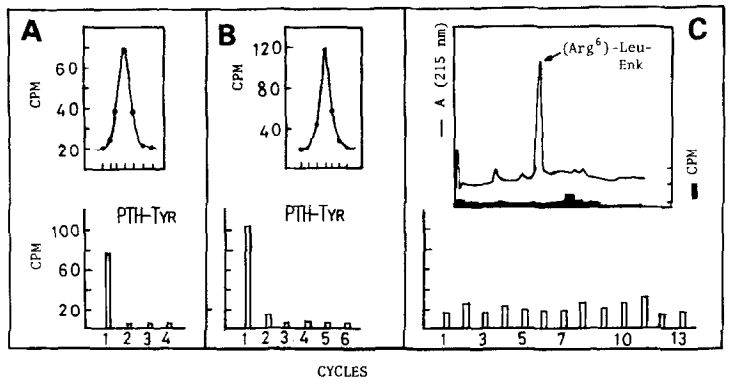

Fig.3. SEQUENCING OF THE ISOLATED PEPTIDES BY EDMAN DEGRADATION. The identity of the PTH-tyrosine residue cleaved from (Arg^6,Arg^7)-Leu-Enk (A), and from (Arg^6)-Leu-Enk (B), and its Gaussian radioactivity distribution (see Inserts), was verified by isocratic HPLC (ODS-3 column; 45% ACN/TFA, pH 3.8; 1.0 ml/min/tube). Radioactivity was found only after the first cycle. The fractions co-sequenced with reference Dynorphin (C) did not show significant radioactivity, as did the hexapeptide (Arg^6)-Leu-Enk, obtained by tryptic digest of Dynorphin (Insert 3C).

Because Liotta and Krieger (8) have already shown the formation of the common precursor of ACTH and β-LPH in the human placenta, our finding of the presence of Met-Enk (i.e. $\beta\text{-LPH}_{61\text{-}65}$) is in itself not surprising. Not previously reported, however, is our original demonstration in this study of the biosynthesis, not only of Leu-Enk, but also of (Arg^6)-Leu-Enk and of (Arg^6,Arg^7)-Leu-Enk in the human placenta. Although the latter heptapeptide constitutes the amino terminal half of Dynorphin (18), there is no evidence of a biogenetic relationship between this tridecapeptide and Leu-enkephalin. In the present study, pure Leu-Enk was obtained in quantities circa 3 times higher than Met-Enk (see Table III and Fig.1, insert), the reverse of what we have found in bovine adrenal medulla (6). However, since we now know that biosynthesis and catabolism of these enkephalins appear to occur concomitantly (cf. Table I), these calculations of Met-Enk versus Leu-Enk ratios must be interpreted with caution. Much more significant in our view is the observation that *de novo* biosynthesis of Met-Enk always appears to be accompanied by formation of its (Leu^5)-counterpart also. In other words, it is not possible to find one without the other. The concept that Met-Enk and Leu-Enk are in

fact fragments of the same precursor molecule, advanced earlier by Lewis, et al. (5) based upon their studies with bovine adrenal medullae, thus finds support in our findings reported in this paper. At present, we can only speculate on the possible physiological role of Leu-enkephalin and related peptides in the placenta, normally not considered an enervated organ.

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