PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 173, 515–518 (1983)

Immunoreactive Met-Enkephalin in the Canine Adrenal; Response to Acute Hypovolemic Stress (41680)

LEO D. FARRELL, TIMOTHY S. HARRISON, AND LAURENCE M. DEMERS[1]

Departments of Pathology and Surgery, The M.S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033

Abstract. Immunoreactive met-enkephalin was measured in the adrenal, kidney, liver, and intestine of the dog using radioimmunoassay. Adrenal tissue concentrations were 20 to 200-fold higher than the other tissues studied. In response to acute hypovolemic stress, the concentration of met-enkephalin in the adrenal vein of the dog increased 6-fold over basal peripheral arterial levels. These results suggest that the canine adrenal gland is a rich source of this opioid peptide and that the adrenal releases met-enkephalin in response to acute stress.

The recent discovery of opiate receptors in the central nervous system has led to intense investigations of the endogenous opioid peptides, their site of synthesis, and their neuropharmacological responses in various organ systems (1). A number of individual opioid peptides have now been isolated and characterized from the central nervous system and peripheral tissues of several species (2, 3).

Hughes and co-workers (2) were the first to characterize the structure of enkephalin, one of the opioid peptides, and showed that it was composed of two pentapeptides, methione-enkephalin (tyr–Gly–Gly–Phe–Met) and leucine-enkephalin (tyr–Gly–Gly–Phe–Leu). It was soon recognized that the amino acid sequence of met-enkephalin was contained within the 61–65th residue of beta-lipotropin, a large 91-amino acid molecule present in the pituitary gland of several species. Beta-lipotropin is cleaved *in vivo* to form two major peptide residues, γ-lipotropin (B-Lpt residues, 1–58) and B-endorphin (B-LPt residues, 61–91). Thus met-enkephalin can be found in the terminal amino acid sequence of B-endorphin. B-endorphin has been shown to have opiate-like activity (2).

It is interesting to note that although met-enkephalin is found within the B-endorphin molecule, its localization in the brain is found in different neurons from those containing B-endorphin (4). This suggests that met-enkephalin may belong to a separate neuropeptide system with its own precursors.

Met-enkephalin has now been localized outside the central nervous system with detection in the kidney, intestines, and adrenal gland of several species accomplished with immunohistochemistry and radioimmunoassay (5–12). Immunocytochemical studies of the rat adrenal gland have localized met-enkephalin to the medullary cells (9). Immunoreactive met-enkephalin by radioimmunoassay has been measured in the adrenal glands of the dog, guinea pig, rabbit, cattle, and rat (10). The canine adrenal gland has been found to contain the highest concentration of met-enkephalin of all the species tested to date (10).

In this study we have determined the tissue concentration of met-enkephalin in the adrenal, kidney, liver, and intestine of the canine and have examined localized canine adrenal artery and venous samples for this peptide following the induction of hypovolemic stress.

Materials and Methods. *Tissue extraction.* Each animal was anesthetized with phenobarbital. The adrenal glands were surgically removed as rapidly as possible and frozen in dry ice. Segments of liver, kidney, and ileum were also removed. The lumenal contents of the ileum were washed out with a cold 0.9% (w/v) NaCl solution (saline). In addition, tissue samples stored in liquid nitrogen were subsequently pulverized, and (0.5–1.0 g) samples were prepared according to the following.

A 10% (w/v) homogenate was prepared and extracted with 0.1 *N* hydrochloric acid at 95°C, with continuous heating for 10 min. The samples were then allowed to cool and particulate matter was removed by centrifugation at 580*g* for 15 min. The supernatants were then lyophilized.

[1] To whom reprint requests should be addressed.

0037-9727/83 $1.50

The lyophilized extracts were then reconstituted with 25 ml of BSA–borate buffer (0.1 *M* borate buffer, 0.1% BSA), and vortex-mixed for 5 min. The buffer was boiled for 5 min to inactivate any enzymatic contamination. The samples were again centrifuged at 580*g* for 10 min and the supernatant was refrigerated.

Canine plasma study. Basal peripheral blood samples (10 ml) were obtained from an anesthetized animal. Hypovolemic stress was induced by exsanguination of 25 ml/kg. After selective catheterization of the left adrenal vein, simultaneous peripheral arterial and adrenal venous blood samples (10 ml) were withdrawn at 15- and 30-min intervals. Plasma was then separated and immediately frozen until assayed. One-milliliter aliquots were withdrawn from each sample and added to 2 ml of 0.1 *N* hydrochloric acid at 95°C, with continued heating for 10 min. Samples were then lyophilized.

The lyophilized extracts were then reconstituted with BSA–borate buffer, vortex-mixed for 5 min, and refrigerated.

Radioimmunoassay. The radioimmunoassay was performed using reagents purchased from Immuno Nuclear Corporation, Stillwater, Minnesota. All reagents were placed in ice and the assay was performed on ice. Standards were prepared in serial dilution by adding 500 μl of a 25-ng/ml met-enkephalin standard solution to 500 μl BSA–borate buffer. This generated standards containing 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, and 0.19 ng/ml. The standard curves were constructed from data on duplicate 200-μl aliquots of the standard solution.

Two hundred microliters of the unknown sample and standards, in duplicate, were transferred to 12 × 75-mm acid-washed glass test tubes. Nonspecific binding (NSB) tubes and zero standard tubes were prepared using 300 and 200 μl, respectively, of BSA–borate reagent. To this we added 100 μl of methionine enkephalin antiserum (diluted 1/900) to the zero, standard, and sample tubes, followed by the addition of ^{125}I-methionine enkephalin to all tubes, including the NSB tubes.

The contents of each tube were vortex-mixed and incubated at 4°C for 16-24 hr. The tubes were then transferred to an ice bath and 100 μl of rabbit gamma globulin added to all tubes. Saturated ammonium sulfate (500 μl) was then used to effect separation of bound from free trace and the tubes were allowed to stand for 15 min. The samples were then centrifuged at 1000*g* for 15 min, at 4°C. The supernatants were decanted and the precipitate of each tube counted in a gamma scintillation counter for 5 min.

The analytical performance of the met-enkephalin radioimmunoassay was thoroughly tested for precision and accuracy through the performance of intra- and interassay precision studies, parallelism, and recovery.

The methodologic coefficient of variation for between run and within run precision was less than 6% at all concentrations tested. Parallelism with the met-enkephalin radioimmunoassay was acceptable over a wide range of dilutions tested with a slope of 3.1, an intercept of 0.187, and a correlation statistic of 0.996. Analytical recovery was performed by adding purified methione enkephalin (Calbiochem-Behring Corp., Somerville, N.J.) to a homogenate of liver tissue and then carrying out the extraction, lyophilization, and radioimmunoassay. Recovery of met-enkephalin throughout the entire procedure exceeded 70% with a correlation value of 0.99 obtained between the concentration of met-enkephalin added and the concentration measured by the radioimmunoassay. The sensitivity of this radioimmunoassay procedure as employed was 38 pg. Cross reactivity with other potentially interferring peptides with the antibody used in this study was minimal. Leucine enkephalin cross-reacted only 1.6%; substance P and Beta-endorphin showed less than 0.5% cross-reactivity; porcine dinorphine (1–13), α-endorphin (61–76) and α-neoendorphin cross-reacted less than 0.002%.

Results and Discussion. A total of 15 pairs of left and right canine adrenal glands, four tissue samples from kidney and ileum, and two tissue samples from liver were analyzed for their met-enkephalin content using the procedures described in this study. Table I depicts these results. There were no significant differences noted between the left and right adrenal gland. The mean value for both adrenal glands was 3219 ± 1266 ng/g of tissue. Although our studies did not involve selectively examining the medulla and cortex sep-

TABLE I. CONTENTS OF MET-ENKEPHALIN[a]

	N	Met-enkephalin	Significance
Adrenal glands	(26)	3219 ± 1266	
Left	(13)	3404 ± 1317	$P = 0.1$*
Right	(13)	3035 ± 1238	
Ileum	(4)	173.25 ± 24.32	$P < 0.01$**
Kidney	(4)	22.25 ± 5.19	$P < 0.01$**
Liver	(2)	14.10 ± 0.71	$P < 0.01$**

[a] Expressed as ng/g wet tissues ± SD.

* The left adrenal gland concentration was found not to be significantly different from the right adrenal gland concentration, using the paired t test ($P = 0.1$).

** Tissue concentrations significantly different from adrenal concentrations, using unpaired two-tailed t test ($P < 0.01$).

arately, Hexum *et al.* (10) have shown that the medulla, particularly the chromaffin granules contained over 90% of the enkephalin-like peptides found in the adrenal gland.

The mean values of met-enkephalin in the kidney, liver, and intestinal (ileum) tissue preparation were significantly lower than the levels found in the adrenal gland ($P < 0.01$). The concentrations of this peptide in these tissues were comparable to values reported by other investigators and support the observation that the adrenal glands are a rich source of this neural peptide. Our finding of 173 ng/g in the ileum of the canine intestine is close to the concentrations reported in the literature for rat and guinea pig ileum and supports the suggestion that the intestines may also be a significant source for met-enkephalin (5).

To document the response of the canine adrenal in terms of met-enkephalin release following an acute stress, we examined the concentration of met-enkephalin in catherized adrenal vein plasma samples from dogs subjected to a hemorrhagic, hypovolemic stressful event, as shown in Fig. 1. The concentration of met-enkephalin in the adrenal vein increased sixfold over the basal peripheral arterial and adrenal vein level within 15 min of the hypovolemic stress from a mean value of 57 pg/ml to 368 pg/ml at 15 min and 357 pg/ml at 30 min. The peripheral arterial concentration also increases significantly at these time points.

These results suggest that met-enkephalin is secreted into the circulation from the adrenal gland with acute stress and support the findings of Govoni *et al.* (15) who noted that stimulation of the splanchnic nerve in the dog results in a voltage-dependent release of met-enkephalin like peptides into the lumbar adrenal vein. It is possible that the dog adrenal releases a variety of enkephalin peptides to a wide range of stimuli some of which are not recognized by our assay. It is clear, however, that hypovolemic stress does provoke a measurable release of met-enkephalin immunoreactive peptides as determined in our study. Other studies (12) with the human adrenal have shown that the human adrenal medulla is particularly rich in met-enkephalin with smaller amounts present in the adrenal cortex. In addition, Schultzberg *et al.* (9) have shown that using immunochemical techniques, enkephalin-like peptides are present in both the adrenal medulla and splanchnic nerve terminals. Thus the possibility exists that the adrenal medulla releases opioid peptides in concert with the catecholamines as part of this

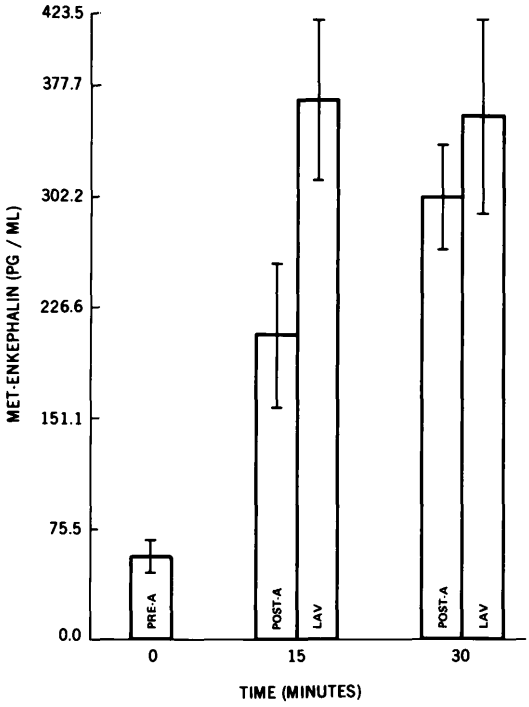

FIG. 1. Canine met-enkephalin secretion study. Prearterial (Pre-A), postarterial (Post-A), and left adrenal vein (LAV) plasma met-enkephalin concentrations ($\bar{x} \pm$ SEM), following hypovolemic stress induced by exsanguination.

glands response to acute stress. Ryder and Eng (14) using a radioimmunoassay for leucine-enkephalin-like material to human and canine plasma recently demonstrated a significant rise of plasma enkephalin in dogs undergoing insulin-induced hypoglycemic stress. Whether these peptides are functioning as a modulator of neural signals outside the central nervous system is unclear at this time and awaits further experimental research. It is clear, however, from our studies that the canine adrenal is particularly enriched in met-enkephalin immunoreactivity and responds to acute stress with a significant release of this opioid peptide.

1. Miller RJ. Ekephalin: a peptide with morphine like properties. Trends Neurosci **1**:29–31, 1978.
2. Hughes J, Smith TW, Kosterlitz HW, Fathergill LG, Morgan BA, Morris HR. Identification of two related pentapeptides from the brain with optent opiate agonist activity. Nature (London) **258**:577–579, 1975.
3. Hughes J, Kosterlity HW, Smith TW. The distribution of methionine enkephalin and leucine enkephalin in the brain and peripheral tissues. Brit J Pharmacol **61**:639–647, 1977.
4. Yang HY, Hong JS, Costa E. Regional distribution of leu and met enkephalin in rats brain. Neuropharmacology **16**:303–307, 1977.
5. Smith TW, Hughes J, Kosterlitz HW, Sosa RP. Enkephalins: Isolation, distribution, and function. In: Kosterlitz H, ed. Opiates and Endogenous Opioid Peptides. Amsterdam, Elsevier/North-Holland, pp 57–62, 1976.
6. Di Giulio AM, Yang HY, Fratta W, Costa E. Decreased content of immunoreactive enkephalin-like peptide in peripheral tissues of spontaneously hypertensive rats. Nature (London) **278**:646–647, 1979.
7. Lewis RV, Stern AS, Rossier J, Stein S, UndenFriend S. Putative enkephalin precursors in bovine adrenal medulla. Biochem Biophy Res Commun **89**(3):822–829, 1979.
8. Yang HY, DiGiulio AM, Fratta W, Hong JS, Majane EA, Costa E. Enkephalin in bovine adrenal gland. Multiple molecular forms of met-enkephalin immunoreactive peptides. Neuropharmacology **19**:209–215, 1980.
9. Schultzberg M, Lundberg JM, Hokfelt T, Terenius L, Brandt J, Elde RP, Goldstein M. Immunoreactivity in gland cells and nerve terminals of adrenal medulla. Neuroscience **3**:1169–1186, 1978.
10. Hexum TD, Yang HYT, Costa E. Biochemical characterization of enkephalin-like immunoreactive peptides of the adrenal gland. Life Sci **27**:1211–1216, 1980.
11. Stern AS, Lewis RV, Kimwa S, Rossier J, Gerber LD, Brink L, Stein S, UndenFriend S. Isolation of the opioid heptapeptides metenkephalin (Arg^6, Phe^7) from bovine adrenal medullary granules and striatum. Proc Natl Acad Sci USA **76**:6680–6683, 1979.
12. Clement-Jones V, Lowry PJ, Rees LH, Besser GM. Met-enkephalin circulates in human plasma. Nature (London) **283**:295–297, 1980.
13. Yoshimasa T, Nakao K, Oht Suki H, Li S, Imura H. Methionine-enkephalin and leucine-enkephalin in human sympathoadrenal system and pheochromacytoma. J Clin Invest **69**:643–650, 1982.
14. Ryder SW, Eng J. Radioimmunoassay of leucine-enkephalin-like substance in human and canine plasma. J Clin Endocrinol Metab **52**:367–369, 1981.
15. Govoni S, Hanbauer I, Hexum TD, Yang HYT, Kelly GD, Costa E. *In vivo* characterization of the mechanisms that secrete enkephalin-like peptides stored in dog adrenal medulla. Neuropharmacology **20**:634–645, 1981.

Received January 10, 1983. P.S.E.B.M. 1983, Vol. 173.