JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 1994, 45, 2, 319–330

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EFFECTS OF NICOTINE ON THE CONCENTRATION OF NATIVE AND CRYPTIC MET- AND LEU-ENKEPHALIN IN PERIPHERAL TISSUES

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The distribution of the native pentapeptides, Met- and Leu-enkephalin, and their cryptic forms (larger enkephalin-containing peptides) in adrenal medulla, spleen, lung, salivary gland, vas deferens, heart, duodenum and jejunum were determined by radioimmunoassay.

The proportion of total Met- and Leu-enkephalin represented by native pentapeptide varied markedly among these tissues. Also, the distribution of native and cryptic Met-enkephalin was distinct from that of Leu-enkephalin.

Repeated short-term administration of nicotine, 0.1 mg/kg i.p. six times at 30 min intervals, produced significant changes in native and cryptic Met-enkephalin in adrenal medulla, jejunum, vas deferens, spleen and heart. This regimen of nicotine also affected the concentration of Leu-enkephalin in adrenal medulla, jejunum and spleen.

Key words: *opiate peptides, nicotine, peripheral tissues, rat.*

INTRODUCTION

Numerous groups have shown evidence for the presence of Met- and Leu-enkephalin in the central nervous system and peripheral tissues (1–4).

Met-enkephalin derived from proenkephalin is present in high concentrations within the adrenal medullary chromaffin cells, heart, pancreas and many other tissues (5, 6). Cosecretion of enkephalin-related peptides and catecholamines has been demonstrated from chromaffin cells *in vitro* (7), isolated perfused adrenal glands (5), adrenal gland *in vivo* (8), during splanchnic nerve stimulation (3, 5) and restraint stress (9, 10).

It has also been shown that Leu-enkephalin may be derived from either prodynorphin or proenkephalin containing neurons (8). The enkephalin and dynorphin systems occur in many of the same brain areas but their distribution in the peripheral tissues is still unknown.

In a number of tissues (brain and peripheral) enkephalin-containing peptides are converted to Met- and Leu-enkephalin in several enzymatic reactions that involve trypsin-like and carboxypeptidase-like enzymes. Met- and Leu-enkephalin derived enzymatically *in vitro* from larger precursors have been referred to as cryptic Met- and Leu-enkephalins. We have established more optimal conditions (4, 10) for the hydrolysis of such larger peptides using trypsin and carboxypeptidase B, than were used previously by others (13 – 15).

Although, the physiological relevance of cryptic enkephalins remains unclear, recently has been reported that plasma cryptic Met-enkephalin increased in response to restraint stress in rats (10). It has also been shown that cryptic forms of Met- and Leu-enkephalin in brain changed under stress conditions (4).

In spite of much investigation of nature of enkephalins, little is known about the cryptic form of these pentapeptides in the peripheral tissues.

Futhermore, aside from the studies of enkephalin interaction with dopamine (1, 16 – 20) understanding of the neuropharmacological regulation of the synthesis, processing and release of the enkephalins is still limited.

In viev of the obvious nicotinic regulation of enkephalin synthesis and release in the adrenal, we have investigated the effects of repeated short-term systemic nicotine administration on the concentrations of native and cryptic (peptidase hydrolyzable) Met- and Leu-enkephalin in a number od selected peripheral tissues.

MATERIALS AND METHODS

Adult, Sprague-Dawley rats (300 – 315 g) were kept individually in an environmental room at 24°C with controlled light-dark cycles (lights on from 0700 – 1900 hr) and provided with free access to food and water.

In order to measure the effect of nicotine on the levels of Met-and Leu-enkephalin in various peripheral tissues, two groups ($n = 7$) of rats were injected with nicotine (0.1 mg/kg i.p.) or saline every 30 min for a total of six injections. Thirty min after the last injection, rats were sacrificed by decapitation. Peripheral tissues (lung, spleen, heart, adrenal medulla, salivary gland, vas deferens, jejunum, duodenum) were dissected rapidly, weighed, homogenized immediately in 10 volumes of 0.5 N HCl containing 0.1% EDTA, and centrifuged at 40.000xg at 4°C for 30 min. Supernatant was lyophilized and stored at −70°C. Lyophilized tissue samples were reconstituted in 1 ml of phosphate buffer and aliquots taken for determination of native and cryptic Met- and Leu-enkephalin. Enkephalin-containing peptides (cryptic enkephalins) were hydrolyzed enzymically with trypsin and carboxypeptidase B as described previously (4). Briefly, we used incubation at 37°C with trypsin 1 mg/g tissue for 30 min, then with carboxypeptidase B 0.05 mg/g tissue plus trypsin inhibitor 2.5 mg/g tissue for 15 min.

Cryptic Met- or Leu-enkephalin concentrations were determined by subtracking the concentration of native Met- or Leu-enkephalin from the concentration of total Met- or Leu-enkephalin present in the aliquot after hydrolysis.

Native and total enkephalins were purified on Porapak columns comprised of 250 mg of Porapak (Waters 80 – 100 mesh) in 3 ml of absolute ethanol. For more details see (10).

Met-enkephalin immunoreactivity was quantitated using commercial antiserum developed in rabbit (Immunonuclear Corp.), ^{125}I-Met-enkephalin (New England Nuclear) and Met-enkephalin standard (Peninsula). The antiserum was used in a final dilution of 1:12,000; it showed cross-reactivities of 100 percent with Met-enkephalin sulfoxide, 2 percent with Leu-enkephalin and <1 percent with Met-enkephalin-Arg-Phe, Met-enkephalin-Arg-Gly-Leu or beta-endorphin. Intra- and interassay coefficients of variation for the assay are 7 and 11 percent, respectively. Recovery of standard Met-enkephalin added to brain homogenate and carried through the entire extraction and radioimmunoassay procedures was 79 percent.

Leu-enkephalin immunoreactivity was quantitated using commercial antiserum developed in rabbit (Immunonuclear Corp.), ^{125}I-Leu-enkephalin (New England Nuclear) and Leu-enkephalin standard (Peninsula) in a total volume of 200 µl. The antiserum was used in a final dilution of 1:15,000 and it showed cross-reactivity of 4% with up to 100 pg Met-enkephalin, considerably different from the 18% reported by the supplier using a different antiserum dilution; the supplier also reported cross-reactivities of 0.6% with dynorpin and less than 0.002% with beta-endorphin, substance P and somatostatin. Fifty percent displacement was 12 pg. Intra- and inter-assay coefficients of variation for the assay are 9 and 14 percent, respectively.

Data were analyzed statistically by Student's t-test (21).

RESULTS

Peripheral tissues concentrations of native and cryptic Met- and Leu-enkephalin

Native Met-enkephalin showed 30-fold range of variation in the peripheral tissues from 0.27 ± 0.03 (heart ventricle) to a maximum in duodenum of 8.44 ± 1.65 pmoles/g tissue (*Table 1*). Cryptic Met-enkephalin showed 40-fold range of variation from 1.92 ± 0.11 to 77.83 ± 8.50 pmoles/g tissue.

The molar ratios of cryptic to native Met-enkephalin ranged from 2.28 in salivary gland to 31.01 in heart atrium (*Table 3*) suggesting that the pentapeptide represents 3 to 43 percent of total hydrolyzable Met-enkephalin in these tissues.

Native Leu-enkephalin showed a 100-fold range variation in the peripheral tissues studied, excluding no detectable amount in heart atrium, whereas cryptic Leu-enkephalin showed a 11-fold range (*Table 2*). The molar rations of cryptic to native Leu-enkephalin ranged from 2.29 in jejunum to 69.30 in adrenal medulla (*Table 3*), suggesting that the pentapeptide represents 1.5 to 43 percent of total hydrolyzable Leu-enkephalin in these tissues.

Effect of nicotine on concentrations of Met- and Leu-enkephalin in peripheral organs

Nicotine administered six times every 30 min, each time at a dose of 0.1 mg/kg i.p. resulted in significant changes of Met-enkephalin in few peripheral tissues. The levels of native Met-enkephalin increased from 4.56 ± 0.72 to 7.51 ± 1.10 pmol/g tissue in jejunum ($P < 0.05$) and decreased

Table 1. Effect of repeated short-term administration of nicotine on native and cryptic Met-enkephalin concentrations in several peripheral tissues

Tissue	Met-enkephalin Concentrations (pmoles/g tissue; Mean ± SE)					
	Control Rats[a]			Nicotine-treated Rats[b]		
	Native	Cryptic[c]	Total	Native	Cryptic	Total
Salivary gland	8.40 ± 0.72	19.10 ± 2.50	27.50 ± 3.00	10.10 ± 1.39	23.11 ± 2.00	33.21 ± 2.76
Adrenal medulla	4.57 ± 0.72	77.83 ± 8.50	82.40 ± 11.60	7.50 ± 1.10*	63.95 ± 7.00	71.45 ± 8.50
Duodenum	8.44 ± 1.65	21.78 ± 3.80	30.22 ± 5.57	7.35 ± 0.52	17.75 ± 2.00	25.10 ± 2.75
Jejunum	4.10 ± 0.29	15.63 ± 2.80	19.73 ± 4.14	5.09 ± 0.33*	13.79 ± 1.80	18.88 ± 3.15
Vas deferens	0.52 ± 0.06	1.92 ± 0.11	2.44 ± 0.14	0.44 ± 0.07	1.35 ± 0.10*	1.79 ± 0.14*
Lung	0.32 ± 0.04	4.94 ± 0.50	5.26 ± 0.64	0.30 ± 0.03	3.56 ± 0.40	3.86 ± 0.57
Spleen	0.92 ± 0.14	9.5 ± 2.00	10.42 ± 2.20	0.60 ± 0.02*	15.60 ± 2.00*	16.20 ± 2.32*
Heart ventricle	0.27 ± 0.03	3.51 ± 0.30	3.78 ± 0.43	0.20 ± 0.04	2.33 ± 0.38*	2.53 ± 0.43*
Heart atrium	0.74 ± 0.15	24.16 ± 2.10	25.00 ± 2.24	0.50 ± 0.07	16.67 ± 2.00*	17.17 ± 2.11*

[a]Control rats received injections of vehicle.

[b]Rats received six injections of nicotine 0.1 mg/kg i.p. at 30 min intervals.

[c]Cryptic Met-enkephalin represents the difference between the total immunoassayable Met-enkephalin after hydrolysis and the native Met-enkephalin.

*Significant difference from corresponding level in control rats at $p < 0.05$.

Table 2. Effect of repeated short-term administration of nicotine on native and cryptic Leu-enkephalin concentrations in several peripheral tissues

Tissue	Leu-enkephalin Concentrations (pmoles/g tissue; Mean ± SE)					
	Control Rats[a]			Nicotine-Treated Rats[b]		
	Native	Cryptic[c]	Total	Native	Cryptic	Total
Salivary gland	3.78 ± 0.55	34.71 ± 5.50	38.49 ± 6.70	3.97 ± 0.26	29.37 ± 2.80	33.36 ± 3.27
Adrenal medulla	0.42 ± 0.07	28.58 ± 3.90	29.00 ± 4.00	0.77 ± 0.08*	26.43 ± 2.40	27.20 ± 2.60
Duodenum	6.22 ± 1.10	20.58 ± 2.10	26.80 ± 4.80	5.10 ± 0.66	18.60 ± 1.50	23.70 ± 2.62
Jejunum	3.62 ± 0.60	8.28 ± 1.90	11.90 ± 2.30	3.27 ± 0.65	2.48 ± 0.30*	6.11 ± 0.80*
Vas deferens	0.30 ± 0.01	8.80 ± 1.00	9.10 ± 1.20	0.24 ± 0.03	7.76 ± 0.80	8.00 ± 1.20
Lung	0.26 ± 0.02	3.04 ± 0.05	3.30 ± 0.70	0.32 ± 0.06	2.35 ± 0.40	2.67 ± 0.60
Spleen	0.66 ± 0.09	8.09 ± 1.90	8.75 ± 2.30	0.39 ± 0.04*	6.53 ± 1.00	6.92 ± 1.26
Heart ventricle	0.06 ± 0.005	4.49 ± 1.80	4.55 ± 2.00	0.06 ± 0.01	3.20 ± 0.50	3.26 ± 0.60
Heart atrium	ND	6.54 ± 0.55	6.54 ± 0.55	ND	6.59 ± 0.55	6.59 ± 0.55

[a]Control rats received injections of vehicle.

[b]Rats received six injections of nicotine 0.1 mg/kg i.p. at 30 min intervals.

[c]Cryptic Leu-enkephalin represents the difference between the total immunoassayable Leu-enkephalin after hydrolysis and the native Leu-enkephalin.

*Significant difference from corresponding level in control rats at $p < 0.05$. ND — no detectable.

Table 3. Molar ratios of Met- and Leu-enkephalin in various peripheral tissues

Tissue	Met/Leu native	Met/Leu cryptic	Met/Leu total	Met cryptic/ native	Met total/ native	Leu cryptic/ native	Leu total/ native
Salivary gland							
Control	2.30	0.62	0.74	2.28	3.28	8.46	10.16
Nicotine-treated	2.64	0.70	0.93	1.97	2.97	7.40	8.41
Adrenal medulla							
Control	11.20	2.76	2.87	17.02	18.05	69.30	69.16
Nicotine-treated	10.00	2.50	2.71	8.50*	9.51*	34.35*	35.35*
Duodenum							
Control	1.40	1.06	1.16	2.50	3.58	3.29	4.30
Nicotine-treated	1.49	0.97	1.09	2.36	3.41	3.62	4.64
Jejunum							
Control	1.17	1.88	1.70	3.68	4.81	2.29	3.29
Nicotine-treated	1.60	4.85*	3.06*	2.70*	3.70*	0.89*	1.94*
Vas deferens							
Control	1.95	0.22	0.27	3.66	4.72	32.30	33.30
Nicotine-treated	1.91	0.20	0.23	3.46	4.02	32.50	33.50
Lung							
Control	1.25	1.67	1.45	18.35	19.37	10.10	11.10
Nicotine-treated	1.35	1.49	1.51	11.11*	12.11*	12.32	13.32
Spleen							
Control	1.44	1.83	1.65	11.25	11.28	8.88	9.88
Nicotine-treated	1.61	2.20	2.17	23.00*	24.00*	16.80*	17.80*
Heart ventricle							
Control	4.84	0.69	0.75	12.77	14.00	88.90	90.00
Nicotine-treated	3.57	0.75	0.80	11.30	12.30	53.80*	55.00*
Heart atrium							
Control	—	—	3.82	31.01	32.30	—	—
Nicotine-treated	—	—	2.69	33.68	34.09	—	—

*Significant difference from corresponding ratio in control rats at $p < 0.01$—0.05.

from 0.92 ± 0.14 to 0.60 ± 0.02 pmol/g tissue in spleen ($P < 0.05$). This fall in spleen was parallel with the increase of cryptic Met-enkephalin from 9.5 ± 2.0 to 15.6 ± 2.0 pmol/g tissue ($P < 0.05$).

Also, this regimen of nicotine decreased cryptic Met-enkephalin in adrenal medulla and total Met-enkephalin in vas deferens, heart ventricle and heart atrium without affecting levels of native and cryptic forms.

No significant changes in native, cryptic and total Met-enkephalin in lung, salivary or duodenum were found when compared to saline controls.

An identical dosage of nicotine caused increase in the levels of native Leu-enkephalin in adrenal medulla from 0.42 ± 0.07 to 0.77 ± 0.08 pmol/g tissue ($P < 0.05$) and decrease in spleen from 0.66 ± 0.09 to 0.39 ± 0.04 pmol/g tissue ($P < 0.05$) (*Table 2*).

Interestingly, nicotine decreased the levels of total and cryptic Leu-enkephalin in jejunum without affecting the native form. Lung, vas deferens, salivary, heart ventricle, heart atrium and duodenum did not show any changes in the native, total and cryptic forms of Leu-enkephalin after nicotine treatment.

Nicotine decreased the molar ratios (*Table 3*) of cryptic/native and total/native Met-enkephalin in the adrenal medulla, lung and jejunum ($P < 0.05$). These ratios were increased by 50% in spleen ($P < 0.05$) after treatment of nicotine.

The molar ratios of cryptic/native and total/native Leu-enkephalin were increased by 50% in spleen and significantly decreased in heart ventricle, adrenal medulla and jejunum after nicotine administration.

Nicotine increased the molar ratios of cryptic and total Met-enkephalin to Leu-enkephalin in the jejunum from 1.88 to 4.85 and from 1.70 to 3.06 ($P < 0.05$), respectively.

DISCUSSION

The opiate peptides, Met- and Leu-enkephalin, are present in the peripheral tissues as native pentapeptides and extended forms which may have opioid activity in their own right or may be precursors of the pentapeptides.

Met-enkephalin is derived from proenkephalin which contains four copies of Met- and one copy of each: Met-enkephalin-Arg-Gly-Leu, Met-enkephalin-Arg-Phe and Leu-enkephalin (5). It has also been shown that in the brain Leu-enkephalin may be derived from either prodynorphin or proenkephalin containing neurons (11). We can presume that the enkephalin and dynorphin systems occur in the same peripheral tissues, but the distribution of the opioid peptides, Met- and Leu-enkephalin, is uneven.

We have found the highest level of native Met-enkephalin in the duodenum followed by salivary gland, adrenal medulla and jejunum.

Much lover concentrations of Met-enkephalin were present in spleen, heart (ventricle and atrium), vas deferens and lung.

The highest level of native Leu-enkephalin was observed in the same tissues – duodenum, jejunum and salivary gland with the exception of the adrenal medulla in which was 10 times lower than that for native Met-enkephalin.

A similar uneven distribution of native enkephalin has been reported in peripheral tissues (2, 6), there are some differences among those reports. The discrepancies may be due to different methods of tissue collection and estimation of enkephalins. These variations may represent differences in reactivities of intermediate peptides to different antisera.

Our results showed that the level of native Met-enkephalin is much higher than native Leu-enkephalin in all peripheral tissues studied, but the molar ratio of Met-enkephalin to Leu-enkephalin varies from 11.20 in adrenal medulla to 1.17 in the jejunum. The level of cryptic Met-enkephalin is higher than that of cryptic Leu-enkephalin in 6 of 9 tissues and lover in 3 of 9 i.e. vas deferens, salivary gland and heart ventricle.

The presence of large amounts of enkephalin-like peptides in the adrenal medulla of different species has been reported (22) but little is known about the cryptic form of enkephalins in the other peripheral tissues.

Using optimal conditions for enzymatic hydrolysis with trypsin and carboxypeptidase B reported previously (4, 10), we found increases of Met-enkephalin to be about 2–4 fold in the vas deferens, salivary gland, duodenum and jejunum; ten fold in the spleen and heart ventricle, eighteen-fold in the lung and adrenal medulla and unexpectedly thirty fold in the heart atrium after peptidase treatment. These differences suggest the highest degree of processing proenkephalin to pentapeptide in the intestine tract and a lesser degree in other tissues. The processing of proenkephalin in the adrenal medulla seems to be less extensive than that in the central nervous system (4) what suggests that the adrenal medulla is not a major Met-enkephalin source or that the synthesis of total Met-enkephalin is increased.

Enzymatic hydrolysis increased immunoreactive Leu-enkephalin by 4-fold in duodenum and jejunum, 10-fold in lung, spleen, salivary, 30 fold in vas deferens and 80 fold in adrenal medulla and heart ventricle. The data suggests that Leu-enkephalin in the vas deferens, adrenal medulla and heart ventricle is derived largely from prodynorphin, whereas Leu-enkephalin in the other tissues studied is probably derived also from proenkephalin.

In spite of very extensive study the neuropharmacologic regulation of peripheral enkephalins remains poorly defined. Nicotine acts at neuromuscular junctions, at autonomic ganglia and in the brain.

Recent studies from a number of laboratories suggest interactions between opioids and nicotine in behavioral and physiological processes (23). Opioids reduce the number of nicotinic receptors on adrenal chromaffin cells and decrease nicotine-induced secretion of catecholamines (24). On the other hand, nicotine increases Met-enkephalin biosynthesis and release and increases the level of Met-enkephalin mRNA in the adrenal, an effect blocked by the nicotinic antagonist, hexamethonium (25).

In our study, nicotine given six times (total dose 0.6 mg/kg) caused increase of native Met-enkephalin concentration without affecting the concentration of cryptic form. It seems probable that this increase reflects increase in processing cryptic forms to intermediate peptides and to Met-enkephalin. Also, it suggests increase in synthesis of proenkephalin.

Eiden et al. (25) have found that exposure to 10 μM nicotine for 24 – 72h resulted in a gradual increase in total Met-enkephalin immunoreactivity in the culture what was preceded by an increase in mRNA coding for proenkephalin. The induction of mRNA by nicotine was rapid – an increase was detectable within 2h of exposure to nicotine and was maximal by 8h. They also reported that secretory products of adrenal (enkephalin, ATP, catecholamines) are maximally released from the cultured cells during the first 15 min of exposure to nicotine. It seems that in our *in vivo* study, nicotine first caused the depletion of intracellular Met-enkephalin due to its release into the periphery and after 3h intracellular peptide stores were higher than the pre-stimulation levels, indicating that compensatory biosynthesis has occured.

Similar increase of only native Met-enkephalin was observed in jejunum, what might be considered that nicotine stimulated the synthesis and processing the proenkephalin related peptides to the pentapeptide. However, it is also possible that nicotine inhibited the release of native Met-enkephalin from jejunum.

This regimen of nicotine seems to have inhibitory effect on synthesis of cryptic Met-enkephalin in vas deferens, heart atrium and ventricle since there was no changes in native form and decrease in cryptic form. It is probable that the dose – 0.1 mg/kg given six times caused desensitization to nicotine. Livett et al. (7) observed in adrenal cells culture that nicotinic induction of enkephalin biosynthesis was maximal at the dose 5 – 10 μM and was not observed with doses of nicotine higher than 1,000 μM. This desensitization to nicotine was also characteristic of nicotine-induced enkephalin and catecholamine release.

Nicotine-induced biosynthesis and Met-enkephalin release was clearly seen in spleen; it was illustrated by significant increase of cryptic and decrease of native peptide.

The effect of nicotine on Leu-enkephalin concentrations in peripheral tissues was less extensive than that of Met-enkephalin. Nicotine might

stimulated the processing of prodynorphin to native Leu-enkephalin in adrenal medulla without increasing its release.

On the other hand, the concentration of native Leu-enkephalin in spleen was much lower after nicotine treatment.

The significant decrease of cryptic Leu-enkephalin in jejunum may reflect increase in release of Leu-enkephalin and processing of prodynorphin without a compensatory increase in synthesis of prodynorphin. It is possible that Leu-enkephalin immunoreactive material found in digestive tract is issue from two different sources: the proenkephalin and the prodynorphin precursors as demonstrated in central nervous system (26, 27).

The ratios of total/native or cryptic/native Met-enkephalin which are higher in heart atrium, adrenal medulla, than in the other peripheral tissues studied, suggest that there is less processing of proenkephalin and its intermediate peptides to the pentapeptide in this tissues.

If nicotine causes the release of Met-enkephalin from adrenal medulla and lung, than processing must also have increased since the ratios of total/native and cryptic/native are lower.

In contrast, the total/native and cryptic/native Met-enkephalin ratios in spleen were decreased after nicotine treatment what suggests increase of proenkephalin synthesis and release of pentapeptide from the tissue.

Ratios of total/native and cryptic/native Leu-enkephalin in vas deferens, heart ventricle and adrenal medulla were considerably higher than ratios of total/native and cryptic/native Met-enkephalin in these tissues, consistent with a hypothesis that prodynorphin is generally less processed than proenkephalin in these tissues.

The significant decreases in the ratios of total/native nad cryptic/native Leu-enkephalin in adrenal medulla heart ventricle, and jejunum after repeated nicotine appear to reflect a decrease in synthesis of precursor and an increase in release of native Leu-enkephalin.

This data showed that the effect of nicotine on the biosynthesis, processing and release of proenkephalin and prodynorphin-derived peptides varied from tissue to tissue.

In order to better define these effects, it will be necessary to measure the effects of nicotine (and other stimuli) on the turnover of synthesis rates (kinetic versus static parameters) of Met- and Leu-enkephalin.

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R e c e i v e d: October 1, 1993
A c c e p t e d: January 7, 1994

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