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Antinociceptive effect of [Met5]enkephalin semicarbazide is not affected by dipeptidyl carboxypeptidase-I

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Dipeptidyl carboxypeptidase-I is an enzyme involved in the biological degradation of enkephalins. It has been suggested that C-terminal amidation of enkephalins enhances their resistance to dipeptidyl carboxypeptidase-I-mediated biodegradation. In this study, a novel [Met5]enkephalin amide (MEA) analogue [Met5]enkephalin (ME)-semicarbazide synthesized by another laboratory in our group was assessed for its antinociceptive effects compared with ME-ethylamide, MEA and ME, using tail flick test. To protect the administered drugs from biodegradation, rats were pretreated with peptidase inhibitors including amastatin, phosphoramidon and captopril. Then captopril (dipeptidyl carboxypeptidase-I inhibitor) was deleted from the peptidase inhibitors' combination for evaluating *in vivo* resistance of the synthetic drugs to dipeptidyl carboxypeptidase-I. According to the results, ME-semicarbazide and MEA were resistant enough to dipeptidyl carboxypeptidase-I to exert their strong antinociception following intrathecal administration even in the absence of captopril, whereas the antinociceptive effects produced by ME-ethylamide (10 nmol) were abolished in rats not pretreated with captopril, indicating that significant amounts of the ME-ethylamide were degraded by dipeptidyl carboxypeptidase-I. Replacement of the amide moiety of MEA with semicarbazide provides a new ME derivative, with high analgesic effects as well as more resistance to dipeptidyl carboxypeptidase-I-mediated biodegradation. **Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.**

Keywords: enkephalin amide; tail-flick latency response; biodegradation; semicarbazide; antinociception; dipeptidyl carboxypeptidase-I

Introduction

As a member of opioid peptides, enkephalins act through three types of opioid receptors, i.e. δ, μ and κ [1]. Morphine, an exogenous ligand of opioid μ-receptor, is still considered as the most effective therapeutic analgesic to manage postoperative or cancer pain [2]. The major problem reported for the long-term administration of morphine is the development of analgesic tolerance [3]. However, intrathecal infusion of some enkephalin derivatives has been shown to restore analgesia in morphine-tolerant patients [4,5]. Enkephalins share some of morphine's positive effects, particularly analgesia [6], whereas their administration results in less adverse effects such as respiratory depression [7,8], constipation [9] and tolerance compared with morphine.

Endogenous enkephalins such as [Met5]enkephalin (ME) and [Leu5]enkephalin are extremely degraded by peptidases, which results in their lower potency compared with morphine [10]. ME is degraded largely by three types of membrane-bound enzymes: amastatin-sensitive aminopeptidase(s), captopril-sensitive dipeptidyl carboxypeptidase-I (angiotensin I-converting enzyme, kininase II) and phosphoramidon-sensitive endopeptidase-24.11 (enkephalinase), in a few minutes (Scheme 1) [10,11].

Naturally, amidation is a mandatory process for the bioactivity of many peptides and, along with other posttranslational modifications, prolongs the half-life of many peptide messengers, protecting them from exopeptidase action in the extracellular space [12].

[Met5]enkephalin amide (MEA) [13] and [D-Ala2-Met5]enkephalin amide [14] are among the early enkephalin amides found to possess higher biological activities and antinociceptive effects than their not-amidated counterparts. Amidation of enkephalins, along with other modifications such as 'D-Ala' substitution at position two (Scheme 1), has produced peptidase-resistant enkephalin analogues (e.g. Tyr-D-Ala-Phe-Met-NH_2), which have been effective enough to provide analgesia following intracerebral administration [15].

It is reported that hydrophobic moieties on C-terminus could further enhance antinociceptive effects of enkephalins [16] as well as their binding affinity to opioid receptors [17–19]. As MEA has been previously identified to possess high biological activity, in this study, the influence of new amide substitutions was examined on its antinociceptive effects and biodegradation by peptidases.

Among the involved peptidases, dipeptidyl carboxypeptidase-I has been demonstrated to be a highly sensitive enzyme to amidation of enkephalin C-terminus, as *in vitro* degradation of

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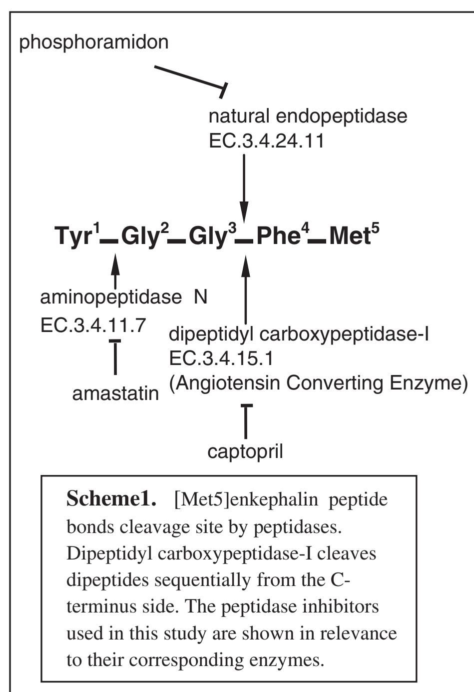

Scheme 1. ME peptide bonds cleavage site by peptidases. Dipeptidyl carboxypeptidase-I cleaves dipeptides sequentially from the C-terminus side. The PIs used in this study are shown in relevance to their corresponding enzymes.

C-terminally amidated enkephalins by this enzyme was largely inhibited, leading to their slower biodegradation [20,21].

Considering the key role of C-terminal moiety of enkephalins on biological activity, in the present study, the antinociceptive effects of a newly synthesized enkephalin derivative, H-Tyr-Gly-Gly-Phe-(Met)-NH-CO-NH_2 [22], was compared with ME, MEA and ME-ethylamide.

The tail flick test has been used to assess the antinocicecptive effects and *in vivo* resistance of the synthetic derivatives to the peptidase-mediated biodegradation following their intrathecal administration in rats. The main aim of this study was to evaluate and compare the resistance of the ME derivatives with dipeptidyl carboxypeptidase-I *in vivo*. Furthermore, the extent to which these ME derivatives exert their antinociceptive effects has been evaluated. Because of the higher *in vitro* sensitivity of dipeptidyl carboxypeptidase-I to ME C-terminal amidation, this study has focused on evaluation and comparison of the *in vivo* resistance of the synthetic ME derivatives with this specific enzyme. Herein, the influence of different substituents on the mentioned parameters has also been assessed.

Methods and Materials

Materials

Peptidase inhibitors (PIs) were provided from the following sources: amastatin, phosphoramidon and captopril were purchased from Sigma-Aldrich (St. Louis, MO, USA). MEA, ME-ethylamide and ME-semicarbazide were synthesized by a combination of solid and solution-phase peptide synthesis using Fmoc and Boc strategy in the 'Peptide Chemistry Research Group, K. N. Toosi University of Technology'. Amidation reaction was carried out by using ammonium chloride, alkylammonium chloride and semicarbazide hydrochloride that led to the formation of amidated C-terminus ME derivatives (Table 1). The molecular weights of these peptides were determined by MALDI MS, and the molecular structures were confirmed using ^{1}H and ^{13}C-NMR.

All chemicals were dissolved in saline as vehicle. The stock solution for all peptides used was prepared at concentrations of 10 mM in siliconized plastic tubes, stored at $-18\,^{\circ}C$ and diluted to the desired concentration just before use.

Animals

Adult male wistar rats (200–250 g) were used in this study. The rats were housed in standard cages on a 12-h light/dark cycle and allowed free access to food and water. The experiments were performed according to the guidelines approved by the Research Committee of Neuroscience Research Center of Shaheed Beheshti University of Medical Sciences in compliance with the standards of the European Communities Council directive (86/609/EEC).

Catheterization of the Spinal Subarachnoid Space

The animals were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylasine (5 mg/kg). To permit the intrathecal administration of drugs into the lumbar subarachnoid space of unanesthetized and unrestrained animals, polyethylene catheters (PE10) were implanted in the L2 and L3 spinal segments, as described previously [23].

Placed on an aluminum platform, the animals' back of the head and neck were shaved. With the use of the ear bars of a stereotaxic holder, the head was clamped flexed forward. A midline incision was made in the skin at the back of the neck, and the muscle was cut at its juncture with the edge of the cranium. The atlantooccipital cisternal membrane was exposed and punctured with a needle to insert an 8.0-cm polyethylene (PE-10) catheter that passed into the intrathecal space.

After a recovery period of at least 1 week, those animals unaffected by the procedure were selected for experiments. On the day of the experiment, the catheter was cut open and used for the intrathecal injections.

PIs and Drug Administration

The mixture of the three following PIs amastatin, captopril and phosphoramidon 10 nmol each was injected 10 min before administration of ME or its derivatives (named as drugs earlier) to inhibit the targeted peptidase(s). In a distinct set of experiments, captopril was then removed from the PIs preparation before intrathecal drug injections. All solutions for intrathecal injections were freshly prepared in normal saline such that the required dose was injected in a volume of 10 µl.

Kanai *et al*. have shown that inhibition of the tail-flick response induced by intra-third ventricular administration of ME was

Table 1. [Met5]enkephalin analogues

Product	$(M+1)^+$
[Met5]enkephalin ethylamide	601.28
[Met5]enkephalin semicarbazide	631.27
[Met5]enkephalin amide	573.25

For details of the synthesis of [Met5]enkephalin amide, ethylamide and semicarbazide, see Arabanian *et al*. [22]. MALDI MS data show the molecular ion peaks $(M+1)^+$ and ^{1}H, and ^{13}C-NMR confirmed the structure.

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augmented by increasing doses of the three mentioned PIs at the dose of 10 nmol each [17]. In our pilot study, intrathecal administration of PIs, each at 10 nmol, resulted in the highest analgesic effects of ME and its derivatives compared with other concentrations. The intrathecal injection was made in a volume of 20 µl (10 µl of drug or PI solution and an additional 10 µl of saline for flushing the drug) and delivered using a 100-µl Hamilton syringes (Hamilton Company, Reno, NV, USA) within 1 min. Considering the increasing latencies of the tail-flick responses in concentrations below 10 nmol, this concentration was chosen for intrathecal drug administrations.

Tail-flick Response

Tail-flick reflex latency was measured using a Tail Flick Analgesia Meter type D-7806 (Hugo Sachs Elektronik, Germany) as described before [24]. A cutoff time set at 10 s was used to prevent damage to the skin on the tail. Latency to flick the tail was measured before and 15, 30, 45 and 60 min after intrathecal injections of ME and its derivatives. Results were calculated as percentage of the maximum possible response: % MPE = 100 × (latency–baseline)/(cutoff–baseline), where cutoff = 10 s. For some experiments, the area under the curve (AUC) value for the antinociceptive action of an opioid on each rat was calculated.

Statistical Analysis

All values were reported as the mean with SEM of the data. Statistical analysis was conducted by using SPSS 16 software for comparison across the experimental conditions. When a significant difference among the groups of AUC data was obtained in the one-way analysis of variance, Dunnett's *post hoc* test was applied to define which group contributed to these differences. The level of statistical significance was set at $p < 0.05$.

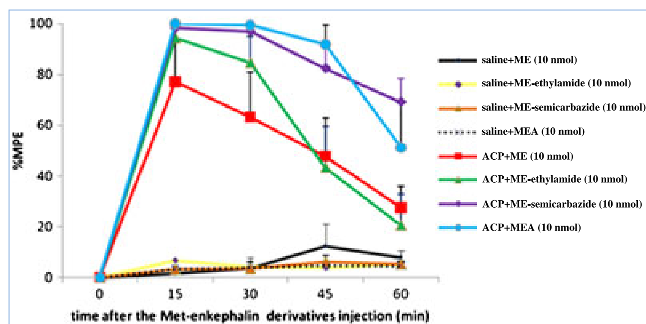

Figure 1. The time course of changes in antinociceptive effects of intrathecal ME and its three amidated analogous MEA, ME-ethylamide and ME-semicarbazide (10 nmol each, $n = 5$ or 6) in rats pretreated and not pretreated with the mixture of the three PIs (ACP) amastatin (A), captopril (C) and phosphoramidon (P). MEA (10 nmol, $n = 5$), ME-ethylamide (10 nmol, $n = 6$) and ME-semicarbazide (10 nmol, $n = 5$). Induced antinociceptive effects by these derivatives in rats are expressed as %MPE. Vertical bars represent the SEM.

Results

MEA, ME-ethylamide and ME-semicarbazide do not Produce Analgesia in the Absence of PIs

The antinociceptive effects of ME derivatives were examined in rats pretreated with the mixture of the three PIs: amastatin, phosphoramidon and captopril. Tail flick responses were evaluated in 5, 10 and 25 nmol of the opioid concentrations, among which 10 nmol was selected. The time course of changes in the inhibitory action on the tail-flick response after the intrathecal administration of 10 nmol of ME, MEA, ME-ethylamide and ME-semicarbazide to rats pretreated intrathecally with three PIs at the doses of 10 nmol each is presented in Figure 1. As it is shown, the observed antinociceptive effects in rats pretreated with three PIs was markedly greater than that in rats not pretreated with any PI.

In agreement with previous reports [25], the PIs by their own did not change the tail flick responses significantly. We did not measure the effect of each PI individually because using a large number of additional animals seemed to be unnecessary considering the main aim of this study, which was to assess resistance of the novel ME-semicarbazide specifically to dipeptidyl corboxypeptidase-I as well as its antinociceptive effects in comparison with known ME derivatives.

Comparing the AUCs of %MPEs in the presence of the three PIs indicated that the amide and semicarbazide derivatives possess higher antinociceptive effects than ME on the tail flick test ($p < 0.01$) (Figure 2). However, there was no significant difference between these two MEAs in their antinociceptive effects ($p = 0.9$). Furthermore, the amide and semicarbazide derivatives seemed to exert more prolonged analgesia compared with ME, as their analgesia remained near the cutoff time for about 45 min after their intrathecal injection. Similar potentiated and prolonged analgesia have been previously reported for the [D-Ala[2]]enkephalin amide [14].

Captopril is not Necessary to Inhibit ME and ME-semicarbazide Hydrolysis *In Vivo*

The tail flick latency responses in rats pretreated with the PIs mixture containing amastatin and phosphoramidon, 10 nmol

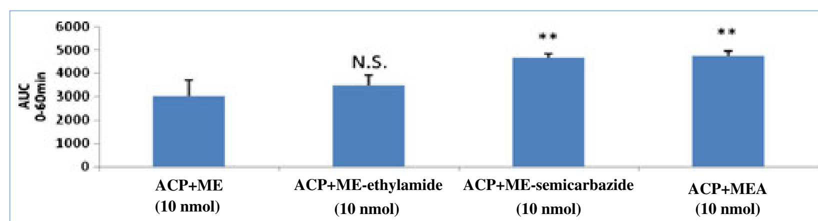

Figure 2. The AUC (0–60 min) values for the ME-induced antinociceptive effect of ME and the amidated derivatives, expressed as %MPE in Figure 2 in rats pretreated with the mixture (ACP) of three PIs. PIs (ACP) amastatin(A), captopril(C), phosphoramidone(P) at the dose of 10 nmol each ($n = 5$ or 6). Vertical bars represent the SEM significantly different from the values of a group that received 10 nmol ME alone by Dunnet's *post hoc* test following one-way ANOVA: $^{**}p \leq 0.01$; NS, not significant.

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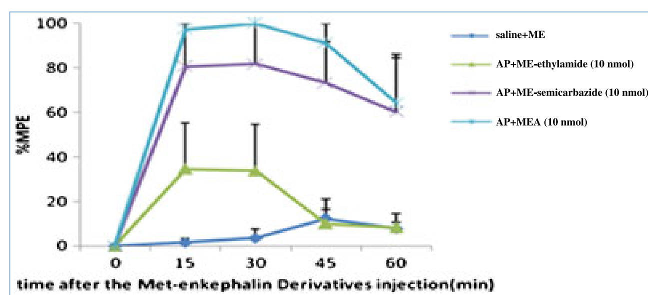

Figure 3. A mixture of two PIs (AP) amastatin (A) and phosphoramidon (P) at a dose of 10 nmol each was injected intratechally 10 min before the intrathecal injection of ME (10 nmol, $n = 6$), MEA (10 nmol, $n = 5$), ME-ethylamide (10 nmol, $n = 6$) and ME-semicarbazide (10 nmol, $n = 5$). Induced antinociceptive effects by these derivatives in rats are expressed as %MPE. Vertical bars represent the SEM.

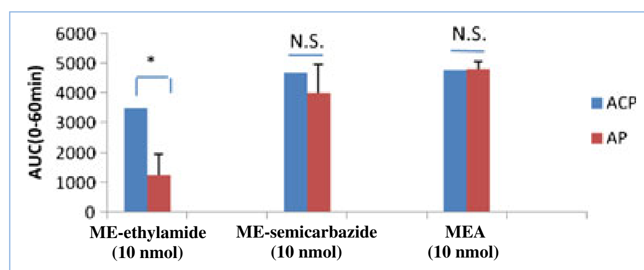

Figure 4. The AUC (0–60 min) values for the ME derivatives induced antinociceptive effects in rats pretreated with the mixture of three (ACP: blue bars) or two (AP: red bars) PIs. PIs: amastatin (A), captopril (C), phosphoramidon (P) 10 nmol each ($n = 5$ or 6) injected intratechally 10 min before the intrathecal injection of ME (10 nmol, $n = 6$), MEA (10 nmol, $n = 5$), ME-ethylamide (10 nmol, $n = 6$) and ME-semicarbazide (10 nmol, $n = 5$). Vertical bars represent the SEM by Dunnett's *post hoc* test following one-way ANOVA: $*p \leq 0.05$; NS, not significant.

each, are represented as %MPE for MEA, ME-ethylamide and ME-semicarbazide in Figure 3. Captopril omission as a specific dipeptidyl carboxypeptidase-I inhibitor in the applied concentrations allows to estimate the degradation of MEs exposed to dipeptidyl carboxypeptidase-I hydrolysis [10].

As shown in Figure 4, the antinociceptive effects of MEA ($p = 0.9$) and ME-semicarbazide ($p = 0.3$), each injected 10 nmol intrathecally, were not attenuated by omitting captopril from the inhibitors' mixture.

There were no significant differences ($p = 0.3$) between the antinociceptive effects of the amide and semicarbazide in rats pretreated with complete inhibitors mixture containing captopril. In contrast, antinociceptive effects of ME-ethylamide dropped off significantly after captopril omission ($p < 0.05$). In these rats, after intrathecal injection of 10 nmol ME-ethylamide, the response latencies in tail flick test disappeared before 45 min (Figure 4).

Discussion

The present study indicates that ME and the amidated derivatives are rapidly degraded *in vivo*. Thus, it seems that pretreatment with the PIs (amastatin, phosphoramidone and captopril) is necessary to determine the precise antinociceptive effects as explained by previous investigations [25–27].

On the basis of our results, MEA and ME-semicarbazide increased the tail-flick latency to the 10-s cutoff time for about half an hour. This implies that these two pentapeptides represent greater and longer-lasting antinociception as compared with ME. The longer-lasting antinociceptive effects may be attributed to more stable bioactive conformation of the enkephalins [28].

It is accepted that C-terminal amidation is one of the effective strategies to produce potent enkephalin analogues [29–31], which some of them produce much stronger analgesia than morphine, as demonstrated in [D-Ala2,MePhe4,Gly]enkephalin amide [30] and Cyclo[Ne,Nb-carbonyl-D-Lys2, Dap5]enkephalin amide [31]. Furthermore, because hydrophobic moieties in the C-terminus could increase tight interactions between enkephalins and the μ-opioid receptor binding site [19], their introduction could result in more potent analgesic analogues [16] such as ME-Arg-Gly-Leu [17], Tyr-D-Ala-Gly-Phe-D-Nle-Arg-Phe [19] and ME-Arg6-Phe7 [18].

The metabolism of endogenously released enkephalins are mainly controlled by two membrane-bound peptidases, which are colocated with opioid receptors within synaptic clefts, i.e. endopeptidase and aminopeptidase N [32,33]. Dipeptidyl carboxypeptidase-I (angiotensin-converting enzyme), however, does not affect the metabolism of endogenous enkephalins significantly [34], but it plays a key role in biodegradation of exogenous enkephalin derivatives before reaching their site of action [32].

It has been shown that amidation of the C-terminal carboxyl group in [D-Ala2-Met5]enkephalin reduced its hydrolysis by natural endopeptidase and also dipeptidyl carboxypeptidase-I. The cleavage activity of dipeptidyl carboxypeptidase-I decreased more drastically as compared with aminopeptidase M [20]. It is consistent with further *in vitro* studies reporting that the presence of a C-terminal amide group greatly reduces the ability of enkephalins to bind to the active site of dipeptidyl carboxypeptidase-I [21,35,36].

In the present study, there was no significant difference between enkephalin amide and semicarbazide tail flick latency responses, even after captopril omission from the inhibitors mixture. Although introduction of a semicarbazide on the C-terminus was expected to weaken the C-terminal ionic interaction with the positively charged active site ($Arg^{\pm}$) of dipeptidyl carboxypeptidase-I [37], our results suggest that enkephalin amide resistance to dipeptidyl carboxypeptidase-I is not be affected by replacement with semicarbazide.

Our results indicate that ethyl substitution significantly abolishes the antinociceptive effects of the amide derivative; however, this hydrophobic group was expected to improve the enkephalin interactions with the μ-opioid receptor binding site [19]. As the antinociceptive effect abolished significantly after captopril omission, it may be suggested that dipeptidyl carboxypeptidase-I plays a key role in proteolysis of the ethylamide derivative *in vivo*.

In conclusion, although several features may be involved in resistance of enkephalins to dipeptidyl carboxypeptidase-I, C-terminal amidation is among the well-demonstrated modifications, resulting in the partial protection of Gly-Phe amide bond as we found in ME-semicarbazide.

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