Antinociceptive effects of des-octapeptide-insulin connected with enkephalins

N. St. Pencheva[1]*, A. I. Bocheva[2], K. Zh. Grancharska[1], T. Barth[3]

[1] Department of Kinesitherapy, South-West University, 66 Iv. Mihailov St., 2700 Blagoevgrad, Bulgaria
[2] Department of Pathophysiology, Medical Faculty, Medical University, 2 Zdrave St., 1431 Sofia, Bulgaria
[3] Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic,
2 Flemingovo nam., 166 10 Praha 6, The Czech Republic

Three insulin analogues with modifications of the B-chain were synthesized by trypsin-catalyzed coupling of des-octapeptide (B^{23}-B^{30})-insulin (DOI) with Met[5]-enkephalin (Met[5]-enk) or Leu[5]-enkephalin (Leu[5]-enk). The derivatives DOI-Met[5]-enk and DOI-Leu[5]-enk were prepared by a condensation between the amino group of the enkephalins and the carboxyl group of arginine in position B^{22}. To test the properties of DOI as a navigating molecule of active opioid peptides, we examined DOI, DOI-Met[5]-enk and DOI-Leu[5]-enk following three types of *in vivo* nociceptive methods: writhing test in mice, paw-pressure test and hot plate test in rats. The peptides were administered both intracerebroventricularly (icv) and subcutaneously (sc). The ability of the peptides to inhibit the electrically-evoked contraction in guinea-pig ileum and mouse vas deferens was also tested. To prove the opioid nature of the responses we used naloxone (1 mg/kg intraperitoneally). Using paw-pressure and hot-plate test, all compounds exerted well-pronounced antinociceptive effects (DOI < DOI-Met[5]-enk < DOI-Leu[5]-enk), with duration at least 40 min after icv application. The differences in order of potency were established after sc application and the effects of all compounds were developed for 20 min. The results obtained with writhing test in mice showed that all compounds did not influence the visceral pain. *In vitro* effects were poor and were observed at concentration higher than 20 μM for DOI-Met[5]-enk and higher than 100 nM for DOI-Leu[5]-enk. The present results suggested that the derivatives of DOI: DOI-Met[5]-enk and DOI-Leu[5]-enk achieved prolonged antinociceptive action, while DOI at some extent could be used as a transport molecule across the blood-brain barrier.

Keywords: des-octapeptide insulin; DOI; Met[5]-enkephalin; Leu[5]-enkephalin; pain; antinociception.

INTRODUCTION

The endogenous opioid pentapeptides Leu[5]-enkephalin (Leu[5]-enk) and Met[5]-enkephalin (Met[5]-enk) and their selective analogues participate in pain control, have analgetic potency and decrease electrically-evoked contractions during *in vitro* assays [1–3]. Leu[5]-enk and Met[5]-enk are released from the polypeptide precursor proenkephalin (267 amino acids), but can not be used as therapeutic drugs, because: (i) the endogenous peptidases degrade them rapidly; and (ii) the so-called peptide transport system 1 [4], which transports enkephalins out of the central nervous system or in both directions, is disputable. However, an artificial precursor of these short opioid peptides was constructed in a previous study of Barth *et al.* [5] by condensing desoctapeptide-insulin (DOI), which molecule is much larger, with Leu[5]-enk or Met[5]-enk. We suggested that the resulting peptides of this condensation could be distributed in the organism and enkephalins would be released from them

gradually with prolonged analgetic action. More-over, the analgetic potency of DOI, as a part of insulin molecule, was also presumed because the antinociceptive effects of insulin are well-docu-mented [6–7] and the painful neuropathy is common in human diabetes [8]. So, the aims of the present study were: (1) to investigate the analgesic proper-ties of DOI, DOI-Leu[5]-enk and DOI-Met[5]-enk, using different *in vivo* tests for antinociception and *in vitro* assays; and (2) to elucidate further if the DOI could be used as a transport molecule across the blood-brain barrier using both – intracerebro-ventricularly (icv) and subcutaneously (sc) appli-cation of the peptides before testing procedures.

EXPERIMENTAL

Animals

The experiments were carried out on male *Wistar* rats (180–200 g), male albino mice ICR strain bred (18–20 g) and male guinea-pigs housed in groups under an artificial 12 h light/dark cycle in air-conditioned room at a temperature of 24 ± 1°C with food and water available *ad libitum* except during

* To whom all correspondence should be sent:
E-mail: nevena_pencheva@yahoo.com

the experiments. Each group included 6–8 animals. All tests were conducted between 09:00–12:00 h.

The following treatment groups were tested for a given peptide: (1) control groups (rats) for NaCl (i.p. 0.9%) after icv or sc application; (2) group (rats) for peptide tested after icv application in volume of 50 μl and activity of 1 IU/kg b.wt.; (3) group (rats) for peptide tested after icv application with naloxone (i.p., 0.5 mg/kg b.wt.) injected 10 min before the test compound; (4) group (rats) for peptide tested after sc application in volume of 50 μl and activity of 1 IU/kg b.wt.; (5) group (rats) for peptide tested after sc application with naloxone (i.p., 0.5 mg/kg b.wt.) injected 10 min before the test compound; (6) group (mice) for acetic acid administered i.p. in the volume of 0.1 ml/10 g b.wt.; and (7) groups for *in vitro* (guinea-pig ileum or mouse vas deferens).

All experimental procedures were carried out in accordance with the institutional guidance and the general recommendations on the use of animals for scientific purposes.

Peptides, drugs, solutions, application

DOI was prepared by trypsin-catalyzed cleavage of porcine insulin as previously described [5]. It was isolated and characterized by mass spectrometry (MS), capillary electrophoresis, amino acid analysis and analytic RP HPLC.

Leu[5]-enk and Met[5]-enk were commercial preparations. DOI-enkephalins (DOI-Leu[5]-enk and DOI-Met[5]-enk) were prepared at the Institute of Organic Chemistry and Biochemistry (Academy of Sciences of the Czech Republic) according to the procedure described previously [5]. The monitoring of the condensation between DOI and enkephalins and the isolation of the products was undertaken by RP-HPLC. The derivatives of DOI were characterized by RP HPLC, capillary electrophoresis, MS-FAB and amino acid analysis.

For *in vivo* and *in vitro* experiments DOI, DOI-Leu[5]-enk and DOI-Met[5]-enk were dissolved in HCl and saline (with a correction of pH) and administered icv or sc. For icv application rats were anaesthetized by i.p. injection of ketamine (80 mg/kg b.wt.) solution for surgical manipulation before application of peptides or NaCl. After sectioning along sagital sature a small hole into the skull was made with the following coordinates from brigma: AP 0.8 mm, ML 1.5 mm and DV 3.5 mm. Rats were allowed 24 h to recover from surgery.

To prove the opioid nature of the responses we used the blocker of the opioid receptors naloxone.

Nociceptive methods (in vivo)

Chemical stimulus - writhing test (or acetic acid-induced abdominal constriction test). Acetic acid (diluted with distilled water to a concentration of 1%) was administered i.p. in the volume of 0.1 ml/10 g b.wt. The mice were placed in individual cages and the number of abdominal constrictions (writhes) of each mouse was counted at 5-min intervals for 30 min. Counting of abdominal constrictions started immediately after injection of acetic acid. The mice with decreased number of writhes were considered protected by the test agent.

Mechanical stimulus - paw-pressure test (or Randall-Selitto test). The changes in the mechanical nociceptive threshold of the rats were measured using an Ugo Basil analgesimeter (probe tip diameter 1 mm). The pressure was applied to the left hind-paw and the pressure (g) required to elicited nociceptive responses such as squeak and struggle was taken as the mechanical nociceptive threshold. A cut-off value of 500 g was used to prevent damage of the paw.

Thermal stimulus - the hot plate test. This test consists of introducing a rat into an open-ended, cylindrical space with a floor consisting of a metallic plate that is heated by a thermode. Hot plate temperature was set at 55 ± 0.5°C. Rats were removed from the hot plate in the absence of nociceptive response within 60 s to avoid the tissue damage. We measure the reaction time or the latency (s) of the first evoked behaviour events such as paw-licking followed by jumping.

In vitro

Male guinea-pigs (200–300 g) or mice were stunned by a blow on the head. The terminal ileum from guinea-pig or vas deferens from mice were removed and placed in the modified Krebs solution containing (mM): NaCl 112.5; KCl 4.75; NaHCO$_3$ 25.00; KH$_2$PO$_4$ 1.19; MgCl$_2$ 1.2; CaCl$_2$ 2.4; glucose 11.5. The segments, 1.5-cm long were dissected out from the ileum, while the length of vas deferens preparations was about 12 mm.

Organ bath experiments. Each ileal segment was set up in an organ bath containing 10 ml of modified Krebs solution aerated with 95% O$_2$ and 5% CO$_2$ and maintained at 37°C. The segments were suspended under 5 mN tension. There was a 60 min equilibration period before any measurements were made. Contractile activity was recorded under isometric conditions. Electrical field stimulation (EFS) (0.5 ms, 0.1 Hz, supramaximal voltage intensity) was applied. Vas deferens preparations were set up

in 3 or 5 ml organ bath with 1 mN tension and the same EFS.

RESULTS AND DISCUSSION

In vivo

Paw-pressure test and hot-plate test – icv application. All compounds DOI, DOI-Leu[5]-enk and DOI-Met[5]-enk exerted well-pronounced antinociceptive effects using paw-pressure test and hot-plate test (Fig. 1). During the paw-pressure assay the pain threshold at the 10-min test interval, which was 120 ± 25 g/cm^2 for the control group, was increased as follows: DOI – to 240 ± 30 g/cm^2, DOI-Leu[5]-enk – to 370 ± 20 g/cm^2 and DOI-Met[5]-enk – to 250 ± 30 g/cm^2. DOI-Leu[5]-enk exerted a maximal antinociceptive potency in the hot-plate assay also, because the maximal latency of the response was 31 ± 5 s at the 20-min interval, while that of DOI and DOI-Met[5]-enk were 20 ± 4 s (at the 20-min and at the 30-min interval) and 20 ± 5 s (at the 30-min interval), respectively.

In the presence of the opioid antagonist naloxone (in a dose of 1 mg/kg; i.p.) the antinociceptive effects of all compounds tested were antagonized (data not shown), which proved their opioid character. Moreover, in the presence of naloxone, the application of the DOI-Leu[5]-enk and DOI-Met[5]-enk, but not of DOI, hyperalgesia was observed.

Paw-pressure test and hot-plate test - sc application. Using this type of injection of the DOI and derivatives we obtain similar antinociceptive effects, but with the following differences (Fig. 2):

– the antinociceptive effect of DOI and DOI-Leu[5]-enk was less revealed than that after icv application;

– the effects of all compounds were developed for 20 min time course, while those after icv – for at least 40 min;

– in the presence of naloxone we did not observed hyperalgesia (data not shown).

It is well known that opioid receptors in the brain modulate descending pain pathways and consequently increase nociceptive response thresholds [9]. Although attenuated at a big extent as compared with icv application, the antinociceptive responses of the DOI and derivatives after sc application suggest that they probably penetrate the blood-brain barrier. However, based on the results with these two tests we need additional experiments to precise the mechanisms of action as far as penetration of blood-brain barrier is concerned.

Writhing test in mice (acetic acid-induced abdominal constriction test). The results obtained with writhing test in mice, which is informative for visceral pain, showed that all compounds did not change the number of the abdominal constrictions after either icv or sc injection. Thus, they did not influence the visceral pain probably because the endogenous enkephalins are rather delta- than mu-selective, while according to Riviere [10] the peripheral kappa-opioid agonists are specific for visceral pain.
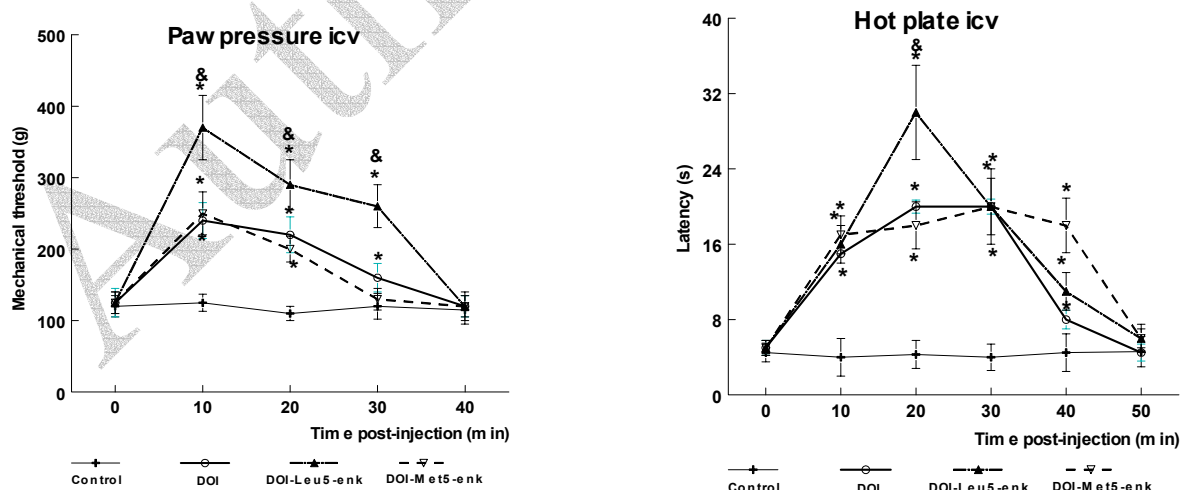

Fig. 1. Antinociceptive effect of icv administrated desoctapeptide-insulin (DOI), DOI-Leu[5]-enkephalin (DOI-Leu[5]-enk) and DOI-Met[5]-enkephalin (DOI-Met[5]-enk) in the paw-pressure and hot-plate test, expressed as mechanical thresholds (*g*) and latencies (s) respectively. Each data point represents the means ± s.e.m. response of 6–8 rats.
*p ≤ 0.05 compared to control by using Mann-Whitney U test; [&]p ≤ 0.05 compared to DOI and DOI-Met[5]-enk by using Mann-Whitney U test.
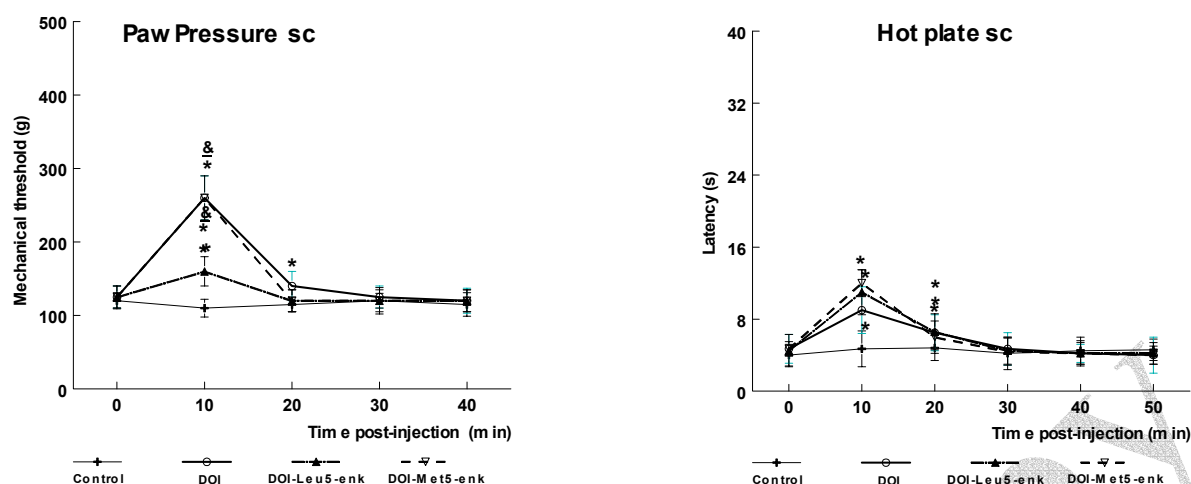
Fig. 2. Antinociceptive effect of sc administrated desoctapeptide-insulin (DOI), DOI-Leu[5]-enkephalin (DOI-Leu[5]-enk) and DOI-Met[5]-enkephalin (DOI-Met[5]-enk) in paw-pressure and hot-plate test expressed as mechanical thresholds (g) and latencies (s) respectively. Each data point represents the means ± s.e.m. response of 6–8 rats.
*$p \leq 0.05$ compared to control by using Mann-Whitney U test; [&]$p \leq 0.05$ compared to DOI-Leu[5]-enk by using Mann-Whitney U test.

In vitro

All three compounds did not affect the electrically-evoked contractions of guinea-pig ileum (mu- and delta-opioid receptors). However, at concentrations higher than 20 µM DOI-Met[5]-enk had naloxone-reversible opioid inhibitory effect, which was about 20% inhibition of the control contractile response.

In the mouse vas deferens (mu-, delta- and kappa-opioid receptors, but predominantly model system for delta-opioid receptors) only DOI-Met[5]-enk showed naloxone-reversible opioid inhibitory effect at concentrations higher than 100 nM. The extent of the effect was about 65% inhibition of the evoked electrical contractions. Since, the inhibitory effects of endogenous enkephalins on electrically-evoked contractions in different smooth-muscle preparations are well documented [1–2], the poor effects of DOI-Leu[5]-enk and DOI-Met[5]-enk could be due to changes in enzyme degradation and in release of the opioid pentapeptides from the condensation peptide in these tissues.

CONCLUSIONS

The present results show that: (i) After mechanical or thermal stimuli DOI, DOI-Leu[5]-enk and DOI-Met[5]-enk exert antinociceptive effects with opioid nature; (ii) The DOI and derivatives do not influence the visceral pain and their *in vitro* effects are also poor; (iii) The antinociceptive effects of the peptides investigated after sc application in both paw pressure test and hot-plate test suggest that they probably have crossed the blood-brain barrier due to DOI, which at some extent could be used as a transport molecule; (iv) The antinociceptive effects of the peptides investigated after icv application in both paw pressure test and hot-plate test suggest that analogues of DOI probably achieved prolonged antinociceptive action, due to gradual release.

REFERENCES

1. N. Pencheva, J. Pospišek, L. Hauzerova, T. Barth, P. Milanov, *Br. J. Pharmacol.*, **128**, 569 (1999).
2. N. Pencheva, P. Milanov, L. Vezenkov, T. Pajpanova, E. Naydenova, *Eur. J. Pharmacol.*, **498,** 249 (2004).
3. N. Pencheva, I. Topouzov, J. Barthova, T. Barth, *Compt. Rend. Acad. Bulg. Sci.*, **55,** 65 (2002).
4. W. A. Banks, A. J. Kastin, *Am. J. Physiol.*, **259**, E1 (1990).
5. T. Barth., L. Klasova, A. Ciencialova., J. Strakova, J. Barthova, V. Kašička, K. Ubik, *Compt. Rend. Acad. Bulg. Sci.*, **55**, 45 (2002).
6. N. Takeshita, S. Yamaguchi, *J. Pharmacol. Exp. Ther.*, **281**, 315 (1997).
7. T. Sasaki, H. Yasuda, K. Maeda, R. Kikkawa, *Neuroreport*, **9**, 243 (1998).
8. Ch. Courteix, M. Bardin, J. Massol, J. Fialip, J. Lavarenne, A. Eschalier, *Neuroreport*, **7**, 1922 (1996).
9. A. I. Basbaum, H. L. Fields, *Annu. Rev. Neurosci.*, **7**, 309 (1984).
10. P. J. Riviere, *Br. J. Pharmacol.*, **141**, 1331 (2004).

141

АНТИНОЦИЦЕПТИВНИ ЕФЕКТИ НА DES-ОКТАПЕПТИД-ИНСУЛИН СВЪРЗАН С ЕНКЕФАЛИНИ

Н. Ст. Пенчева[1]*, А. И. Бочева[2], К. Ж. Грънчарска[1], Т. Барт[3]

[1] *Катедра „Кинезитерапия", Югозападен университет, ул. „Иван Михаилов" № 66, Благоевград 2700,*
[2] *Катедра „Патофизиология", Медицински факултет, Медицински университетет,*
ул. „Здраве" № 2, София 1431,
[3] *Институт по Органична химия и биохимия, Аакадемия на науките на Чешката република,*
Флемингово 2, 166 10 Прага 6, Чешка република

(Резюме)

Три инсулинови аналога с модификации на В-веригата бяха синтезирани чрез трипсин-катализирано куплиране на des-октапапептид (B^{23}-B^{30})-инсулин (DOI) с Met^5-енкефалин (Met^5-enk) или Leu^5-енкефалин (Leu^5-enk). Аналозите DOI-Met^5-enk и DOI-Leu^5-enk бяха получени чрез кондензация между амино групите на енкефалините и карбоксилната група на аргинина в позиция B^{22}. За да тестваме свойствата на DOI като транспортна молекула за активни опиоидни пептиди, ние изследвахме DOI, DOI-Met^5-enk and DOI-Leu^5-enk, чрез три типа *in vivo* ноцицептивни методи: writhing тест на мишки, paw-pressure тест и hot plate тест на плъхове. Пептидите бяха приложени по два начина: интрацеребровентрикуларно (icv) и подкожно (sc). Способността на пептидите да инхибират електрически-предизвикани контракции на илеум от морско свинче и vas deferens от мишка, също беше изследвана. За верификация на опиодината природа на отговорите използвахме налксон (1 mg/kg, интраперитонеално). Приложението на paw-pressure и hot-plate теста показа, че всички съединения упражняваха добре изразени антиноцицептивни ефекти (DOI < DOI-Met^5-enk < DOI-Leu^5-enk) с продължителност от минимум 40 min след icv приложение. Бяха установени различия в потентността на пептидите при sc приложение и ефектите се развиваха за 20 min. Резултатите от writhing теста на мишки показаха, че всички изследвани пептиди не повлияват висцералната болка. Ефектите in vitro бяха твърде слаби и се наблюдаваха при концентрации над 20 μM за DOI-Met^5-enk и над 100 nM за DOI-Leu^5-enk. Въз основа на представените експериментални резултати може да се направи предположението, че аналозите на DOI: DOI-Met^5-enk and DOI-Leu^5-enk проявяват пролонгирано антиноцицептивно действие, а DOI в някаква степен може да играе роля на транспортна молекула през кръвно-мозъчната бариера.