Peptides 36 (2012) 9–16

Contents lists available at SciVerse ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

LVV-hemorphin 7 and angiotensin IV in correlation with antinociception and anti-thermal hyperalgesia in rats

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ARTICLE INFO

Article history:
Received 16 February 2012
Received in revised form 20 March 2012
Accepted 20 March 2012
Available online 29 March 2012

Key words:
LVV-hemorphin 7
Angiotensin IV
Insulin-regulated aminopeptidase (IRAP)
Hyperalgesia
Carrageenan
Spinal cord

ABSTRACT

Hemorphins, a family of atypical endogenous opioid peptides, are produced by the cleavage of hemoglobin β-chain. Hemorphins were proved to bind to the μ-opioid receptors (agonist) and angiotensin IV receptors (insulin-regulated aminopeptidase; IRAP) (inhibitor). Among the hemorphins, LVV-hemorphin-7 (LVV-H7) was found to be abundant and with a longer half life in the CNS. Using intrathecal and intracerebroventricular injections, LVV-H7 and angiotensin IV were given to the rats, which were then subjected to the plantar test and the tail-flick test. Our results showed that LVV-H7 attenuated carrageenan-induced hyperalgesia at the spinal level, which could not be reversed by the co-administration of naloxone. At the supraspinal level, LVV-H7 also produced a significant anti-hyperalgesia effect but with a lower extent. Angiotensin IV showed a similar anti-hyperalgesia effect at the spinal level, but had no effect at the supraspinal level. In the tail-flick test and paw edema test, both peptides showed no effect. These results suggest that LVV-H7 mainly exert the anti-hyperalgesia effect at the spinal level, possibly through IRAP but not μ-opioid receptors. In addition, we observed the expression of IRAP in the CNS of animals with/without carrageenan-induced hyperalgesia. Our results showed a significant expression of IRAP in the spinal cord of rats. However, there was no significant quantitative change of IRAP after the development of hyperalgesia. The serum level of LVV-H7 was also found to be with no change caused by hyperalgesia. These results indicated that the endogenous LVV-H7 and IRAP may not regulate the severity of hyperalgesia through a quantitative change.

1. Introduction

Hemorphins were first identified as a family of novel opioid peptides derived from hemoglobin [5]. By the cleavage of hemoglobin β-chain, many hemorphin peptides with different length could be produced *via* various enzymatic digestions including pepsin, chymotrypsin-like proteases, macrophagic enzymes, lysosomal proteases, and aspartic endoprotease cathepsin [9,12–14,34]. Among the hemorphins with different length, LVV-hemorphin-7 (LVV-H7; Leu–Val–Val–Tyr–Pro–Trp–Thr–Gln–Arg–Phe) is the longest with 10 amino acids. Many reports indicated that LVV-H7 was most abundant with the highest hydrophobicity in mammalian CNS [19,30]. LVV-H7 was first found to bind to opioid receptors in spite of its low affinity [5]. Many of the biological activities (*e.g.* antinociception and hypotention) by LVV-H7 were thought to be mediated by activation of μ-opioid receptors [4,25]. However, recent investigations indicate that the major target of LVV-H7 should be insulin-regulated aminopeptidase (IRAP), since its binding affinity to IRAP is much higher. By blocking IRAP, LVV-H7 may exert its actions in CNS, especially to enhance memory in the hippocampus [2,22,23].

In addition to LVV-H7, angiotensin IV (Ang IV) was also proved to bind to IRAP as a blocker [1]. Ang IV (Val–Tyr–Ile–His–Pro–Phe) was produced from the cleavage of angiotensin III (Ang III) and originally supposed to be the end product of renin-angiotensin system. Instead of being with no function, Ang IV was found to be an endogenous IRAP blocker and thereby caused a significant effect on enhancing/improving memory [6,23,33]. In 2004, Stragier et al. reported that angiotensin II tended to transform to Ang IV more than to stop at angiotensin III in neurons [36]. This highlights the possible biological importance of Ang IV in mammalian CNS, *via* blocking IRAP.

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http://dx.doi.org/10.1016/j.peptides.2012.03.019

As to the functions of IRAP in CNS, IRAP was first identified as a membrane-bound aminopeptidase, which belonged to the same family of Zn^{2+}-dependent amimopeptidases as AP-N and AP-A [1,35]. In humans, oxytocinase (otase) was found to be with 87% homology of amino acid sequence with IRAP, therefore otase was recognized as a subtype of IRAP [16,18,21,28,35]. Otase was also called placental leucine aminopeptidase (P-LAP), which could degrade circulating oxytocin to prevent premature delivery in pregnant women [17,31,40]. IRAP was widely distributed in many tissues, including brain. IRAP was identified to be located in the cortical regions, hippocampus, amygdala, thalamus, hypothalamus, and many other brain regions [1,11,27]. In 1995, Moeller et al. demonstrated that the binding sites of Ang IV (IRAP) were present in the dorsal root ganglia (DRG) and the lamina II of dorsal horn in the spinal cord [29]. This implied a possible functional role of IRAP in regulation of nociception at the spinal level.

Apart from the endogenous inhibitors, Ang IV and LVV-H7, the substrates of IRAP were found to be many endogenous peptides, including Ang III, oxytocin, vasopressin, Lys-bradykinin, Met-enkephalin, dynorphin A, neurokinin A, neuromedin B, somatostatin, and cholecystokinin-8 [15,16,24,27]. One of these has been evidenced by the experiments in IRAP knock-out mice, which has a higher level of vasopressin in blood [39]. Therefore, the activity of IRAP should be important in the regulation of the level of its peptide substrates, especially oxytocin and vasopressin. The possible effects of Ang IV and LVV-H7 on nociception and hyperalgesia could be *via* their blockade of IRAP to increase the level of IRAP substrates.

In the present study, we attempted to investigate the possible effects of Ang IV and LVV-H7 on nociception and hyperalgesia in adult male rats. Using the tail-flick test and the plantar test in carrageenan-induced hyperalgesia model, the effects of Ang IV and LVV-H7 were examined at the supraspinal and the spinal level. The possible effects of LVV-H7 on opioid receptors were also examined with the co-administration of naloxone. The major pharmacological target of LVV-H7 and Ang IV, IRAP, was tested for its quantitative change in the spinal cords during carrageenan-induced inflammation of the paw. This could be the first report of the possible anti-hyperalgesia caused by LVV-H7 and Ang IV, which could be of great importance in the exploration of basic biomedical research and future clinical application of these two peptides.

2. Material and methods

2.1. Animals

Adult male Sprague-Dawley (S.D.) rats were used in the present study. The rats were purchased from BioLASCO Taiwan Co., Ltd. with the body weights between 300 and 400 g. All animals were bred in the Animal Facility of the National Defense Medical Center. The animal rooms were maintained at $23 \pm 2\,^{\circ}C$ with a 12-h light/dark cycle. Food and water were available *ad libitum* throughout the experiment. Animals were taken to the testing room in the morning of the experiment; the experiments were carried out during the light cycle. The experimental protocol was approved by the Animal Care and Use Committee of the National Defense Medical Center, Taipei, Taiwan, ROC.

2.2. Peptide synthesis

Angiotensin IV and LVV-hemorphin 7 were synthesized manually on a solid phase support as described [37]. Standard Fmoc-strategy was employed in the synthesis. N-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids were coupled to a Wang resin (Novabiochem, Switzerland) using *N*,*N*′-diisopropylcarbodiimide (DIPC) with 4-dimethylaminopyridine (DMAP) as the coupling reagent for the first amino acid. The following couplings of amino acids were performed in dimethylformamide (DMF) using benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate (BOP) and N-hydroxybenzotriazole (HOBt). Synthesized crude peptides were cleaved from the resin using 97.5% trifluoroacetic acid in water. Gel filtration (Sephadex G-10; Pharmacia, Piscataway, Sweden) was performed to desalt the crude peptides. The purity of the peptide was checked by analytical reverse-phase high-performance liquid chromatography (HPLC) (RP-HPLC) using a Beckman HPLC system (System Gold) coupled to a UV detector (Gilson, Middleton, WI, U.S.A.). Purification was carried out by HPLC, if required.

2.3. Implantation of an intrathecal catheter and an intracerebroventricular guide cannula

The procedures of surgeries to implant the intrathecal (i.t.) catheter and the intracerebroventricular (i.c.v.) guide cannula were following the methods in our previous reports [7,8]. Before the surgeries, rats were anesthetized with pentobarbital (50 mg/kg, i.p.). An intrathecal catheter was implanted at the lumbar level for drug administration as previously described [7,8]. For implantation of an intracerebroventricular cannula, a stainless-steel cannula (thin-walled, 23-gauge stainless-steel tube) was implanted into the left lateral ventricle according to the coordinates: p +1.0 mm, L +1.25 mm, and V −4–5 mm using the bregma as the origin [32]. The cannula was firmly attached to the skull with two metal screws and dental cement. Each animal was allowed 4 days to recover from the surgery, and was not used for more than one experiment. Any rat showing motor impairment was not employed in the following study. One day before the experiments, animals with intrathecal catheters were injected with 20 μl of 2% lidocaine in a microsyringe (Hamilton, 25 μl) to induce a 10–20 min of temporary motor blockade of the lower limbs and checked whether the catheter was in the correct position. For animals with the intracerebroventricular guide cannula, the position of the cannula was confirmed by negative pressure from the lateral ventricle, which caused 5 μl of saline in the catheter connected to the cannula to fall freely by gravity.

2.4. Drug administration

The peptides and drugs to be administered were dissolved in saline to make the concentration for the desired dose. The volumes for i.t. and i.c.v. injections were both fixed as 10 μl. In i.t. administrations, 10 μl of saline was given to flash out the drugs remained in the tubing following each injection. Insulin syringe (0.3 ml) was employed in manual i.t. injections. In i.c.v. administrations, drugs or saline (10 μl) were delivered with a microdialysis pump (CMA, Sweden). The flow rate was set at 2.5 μl/min. After 4 min, the pump was stopped and the cannula stayed for one more min. to ensure that the drugs will not leak out by the back pressure.

2.5. Plantar test in carrageenan-induced inflammation

To induce acute inflammation, 100 μl of carrageenan type IV (Sigma, U.S.A.) (1.5%, w/v saline) solution was injected into the subcutaneous space of right hind paw of rats. Following the injection of carrageenan, i.t. or i.c.v. injections were performed promptly in different groups of rats. An Ugo Basile 7371 plantar tester (Italy) was used to measure the paw withdrawal latency of the paw received carrageenan injection. The IR intensity was set at 45 and the cut-off time was 20 s. The basal latency was measured before the intraplantar injection of carrageenan (−1 h). The other time points for the determination of paw withdrawal latency were 0 h, 1 h, 2 h,