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Analgesic effects of intra-nasal enkephalins

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Abstract

The analgesic effects of intranasal delivery of leucine enkephalin (Leu-Enk) and its synthetic analogue [D-ala^2]-leucine enkephalinamide (YAGFL) with or without enzyme inhibitors and/or absorption enhancers were investigated using the acetic acid-induced writhing test in mice. The analgesic activity was significantly affected by the time delay after the administration of Leu-Enk; the inhibition rates for the groups administered with acetic acid 5 min and 30 min after the administration of Leu-Enk were 56.40 ± 8.54 and 17.98 ± 7.07%, respectively. The addition of enzyme inhibitors and absorption enhancers markedly increased the inhibition rate of Leu-Enk and YAGFL; their inhibition rates were about four times and twice those without any enzyme inhibitor or absorption enhancer, respectively. The enzyme inhibitors and absorption enhancers that produced the highest inhibition rates of Leu-Enk and YAGFL were azelaic acid (1%), thimerosal (0.5 mM, TM), ethylenediaminetetraacetic acid (5 mM, EDTA) and L-α-lysophosphatidylcholine (0.5%, LPC), and TM (0.5 mM), EDTA (5 mM), LPC (0.5%) and povidone (5%), respectively. The ED50 value of both enkephalins was also determined and found to be about 13 μg kg^{-1}, which is 850 and 60 times more potent than literature values for ketoprofen and morphine, respectively. Based on these results it was concluded that Leu-Enk or YAGFL could exert very high analgesic activity when administered nasally with a combination of inhibitors and absorption enhancers as compared with other analgesics.

Introduction

Enkephalins are pentapeptides that are known to be neurotransmitters in pain transmission (Jankovic & Maric 1988). In addition, they produce other pharmacological actions, such as modulation of blood pressure and modification of morphine withdrawal (Dzoljic & Dzoljic 1989). With the recent advances in recombinant DNA technology, peptide and protein drugs have become available. To be applied successfully in therapy, however, they must be able to reach the site of action and maintain their pharmacological activities for a sufficient length of time. Like other proteins and peptides, enkephalins have extremely low oral bioavailability, mostly because they are rapidly inactivated by various peptidases in the gastrointestinal tract. Thus, their analgesic actions are very short by the oral route and their half-lives are less than 1 min (Frederickson & Geary 1982). Therefore, for systemic delivery, the parenteral route is considered to be the primary candidate for administration.

To overcome the limitations of oral delivery and the inconvenience of parenteral administration, several attempts have been made to deliver enkephalins through the nasal (Hussain et al 1989; Faraj et al 1990a,b; Hussain et al 1995), ocular (Stratford et al 1988), intestinal (Kerchner & Geary 1983) and dermal (Choi et al 1990) routes. Among these, the nasal route has been investigated most frequently for systemic delivery of peptide and protein drugs, including enkephalins.

Nasal delivery has the following general advantages: the avoidance of degradation in the gut and the reduction of first-pass metabolism by oral administration, the potential of an extensive absorption surface in the nose, the relatively rapid systemic circulation, and the possibility of better patient compliance when compared with parenteral therapy (Fisher et al 1991). However, polypeptides are poorly absorbed from the nasal cavity (O'Hagan et al 1990) and are also extensively degraded in the mucosal cavities (Chun & Chien 1993). Several specific enzymes have been suggested as being responsible for the metabolism of enkephalins (Schwartz et al 1981). To inhibit

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degradation by enzymes and enhance absorption, absorption enhancers and peptidase inhibitors have been investigated (Su et al 1985; O'Hagan et al 1990; Sayani et al 1993, 1994; Chun & Chien 1995; Chun et al 1996a).

In a previous study we examined the effect of enzyme inhibitors and absorption enhancers on the in-vitro permeation of leucine enkephalin (Leu-Enk) and its synthetic analogue [D-ala^2]-leucine enkephalinamide (YAGFL) across the nasal mucosa (Gwak et al 2002). Based on the results, in this report we investigate the analgesic effects of nasally administered Leu-Enk and YAGFL with or without enzyme inhibitors and/or absorption enhancers, and compare the analgesic potency with that of a conventional analgesic drug, ketoprofen. To achieve this objective, the acetic acid-induced writhing test was used. The ED50 values of Leu-Enk and YAGFL were also obtained to compare them with those of other analgesics.

Materials and Methods

Animals

ICR mice weighing 20–25 g were purchased from Seoul National Hospital Animal Laboratory Service (Seoul, South Korea). All experimental procedures involving laboratory mice in the present study conformed to the NIH Guide for the Care and Use of Laboratory Animals.

Materials

Leu-Enk, YAGFL, amastatin hydrochloride (AM), thimerosal (TM), ethylenediaminetetraacetic acid (EDTA), azelaic acid (AA), L-α-lysophosphatidylcholine (LPC) and acetic acid were purchased from Sigma Chemical Co. (USA). Povidone (Kollidon 30; BASF, Germany) and ketoprofen (Hongsung Pharm. Co., Korea) were of pharmaceutical grade.

Leu-Enk solution preparation

Fifty microlitres of Leu-Enk (1000 μg mL^{-1}) was mixed with 125 μL of AM (0.5 mM), 25 μL of TM (10 mM) and 25 μL of EDTA (0.1 M) as enzyme inhibitors, and 250 μL of LPC (1%) as an absorption enhancer, and then 25 μL of normal saline was added, resulting in the final concentrations of Leu-Enk (100 μg mL^{-1}), AM (125 μM), TM (0.5 mM), EDTA (5 mM) and LPC (0.5%). For another preparation, AA was used at a final concentration of 1% instead of AM, and the other substances were used at the same concentrations as above.

YAGFL solution preparation

Fifty microlitres of YAGFL (1000 μg mL^{-1}) was mixed with 25 μL of TM (10 mM) and 25 μL of EDTA (0.1 M) as enzyme inhibitors, and then 400 μL of normal saline was added, resulting in the final concentrations of YAGFL (100 μg mL^{-1}), TM (0.5 mM) and EDTA (5 mM). In another preparation, 50 μL of YAGFL (1000 μg mL^{-1}) was mixed with 25 μL of TM (10 mM) and 25 μL of EDTA (0.1 M) as enzyme inhibitors, and 250 μL of LPC (1%) as an absorption enhancer, and then 150 μL of normal saline was added, giving final concentrations of YAGFL (100 μg mL^{-1}), TM (0.5 mM), EDTA (5 mM) and LPC (0.5%). Povidone, at a final concentration of 5%, was added to the last preparation.

Nasal administration

The mice were lightly exposed to ether vapor to induce sedation, avoiding the influence of ether on the writhing as far as possible before the experiments. The mice were intra-nasally administered with normal saline and 5 μL of various test peptides per 20 g (body weight) for the control and test groups, respectively, using a 50 μL Hamilton microsyringe to prevent sneezing during administration. Ketoprofen solution (0.1%) in pH 7.4 isotonic phosphate buffer was administered as a reference drug at a volume of 0.2 mL per 20 g (body weight) by oral route using a sonde.

For the study of Leu-Enk, mice were divided into four groups and Leu-Enk solution preparations at a volume of 5 μL per 20 g (body weight) were administered as follows: administration of Leu-Enk alone 5 min before the administration of acetic acid (L1); administration of Leu-Enk alone 30 min before the administration of acetic acid (L2); administration of the preparation with mixed enzyme inhibitors, including AM, and an absorption enhancer 30 min before the administration of acetic acid (L3); administration of the preparation with mixed enzyme inhibitors, including AA, and an absorption enhancer 30 min before the administration of acetic acid (L4).

For the study of YAGFL, mice were also divided into four groups and administered with YAGFL solution preparations 30 min before the administration of acetic acid at a volume of 5 μL per 20 g (body weight): administration of YAGFL (100 μg mL^{-1}) alone (Y1); administration of the preparation with enzyme inhibitors (Y2); administration of the preparation with enzyme inhibitors and LPC (Y3); administration of the preparation with enzyme inhibitors, LPC and povidone (Y4).

For dose-dependence studies, Leu-Enk with TM (0.5 mM), EDTA (5 mM), AM (125 μM) and LPC (0.5%) was administered at doses of 2.5, 12.5, 25 and 75 μg kg^{-1}, respectively, while YAGFL with TM (0.5 mM), EDTA (5 mM) and LPC (0.5%) was administered at the same doses as Leu-Enk. The ED50 values were calculated from the dose–response curves obtained from the intranasal administration of Leu-Enk and YAGFL.

Acetic acid-induced writhing test

Acetic acid (1.0%) at a volume of 0.2 mL per 20 g (body weight) in distilled water was intra-peritoneally injected into mice. The number of writhings occurring between 5 and 15 min after acetic acid injection was counted. The values from enkephalin-treated groups were compared with those of the control and ketoprofen groups. In this study, a group of seven mice were used and fasted overnight for each preparation. When any mouse did not

respond, the animal was discarded. Whenever an additional test was needed, one group of seven mice was adopted on the same day.

Calculation of inhibition rate

Inhibition rate (%) = (writhing number of control group − writhing number of test group)/writhing number of control group × 100.

Statistics

The one-way ANOVA was employed for simultaneous statistical analysis of three or more groups. For individual differences between groups, Dunnett's and Duncan multiple range tests were used.

Results

Effect of the time after the administration of Leu-Enk

It has been reported that analgesic activity of Leu-Enk remained 2–6 min after administration and disappeared rapidly after 10–12 min (Belluzzi et al 1976). We investigated the effect of the amount of time after the administration of Leu-Enk on the writhing response. As shown in Table 1, the inhibition rate for L2 was very low (17.98 ± 7.07%). However, L1 showed a very high inhibition rate of 56.40 ± 8.54%, which was close to that of ketoprofen (50.73 ± 7.61%). This was possibly due to the extensive degradation of the peptide in nasal mucosa as time passed.

Effect of enzyme inhibitors and absorption enhancers on the analgesic effect of Leu-Enk and YAGFL

As shown in Table 1, the inhibition rate of Leu-Enk increased markedly when enzyme inhibitors and an absorption enhancer were added in the solution preparation; their inhibition rates were about four times that without any enzyme inhibitor or absorption enhancer. Their inhibition rates were more than that of the 5-min group (L1). There were statistically significant differences between means ($P < 0.01$). The effects of the enzyme inhibitors employed in this study on the nasal delivery of peptide have been demonstrated in earlier studies (Sayani et al 1993; Gwak et al 2002). The addition of the mixed inhibitors of AM or AA, EDTA and TM showed a dramatic decrease in the Leu-Enk degradation rate in the rabbit nasal mucosa extracts, about 40-fold in both mucosal and serosal mucosa (Sayani et al 1993).

As shown in Table 2, YAGFL alone showed a significant inhibition effect of 35.5%. The inhibition rate was enhanced greatly with the addition of TM and EDTA. Even though the addition of LPC failed to show a significant increase in the inhibition rate, the further addition of povidone increased it by 14%; their inhibition rates were almost twice that of YAGFL alone. The highest inhibition rate was about 70%, similar to that of Leu-Enk with enzyme inhibitors including AA and LPC; it was 20% more than that of ketoprofen. It was also found that there were statistically significant differences between means ($P < 0.01$).

Effect of dose on the analgesic effect of Leu-Enk and YAGFL

Leu-Enk with TM, EDTA, AM and LPC and YAGFL with TM, EDTA and LPC significantly and dose-dependently inhibited the writhing responses of mice. Linear curves were obtained ($y = 20.237\ \ln(x) - 1.2911$; $R^2 = 0.9982$ and $y = 19.288\ \ln(x) + 0.7379$; $R^2 = 0.9873$ for Leu-Enk and YAGFL, respectively). From the curves, the ED50 values of Leu-Enk and YAGFL were calculated to be 13.35 and 12.98 $\mu g\,kg^{-1}$, respectively, which are very similar. This result demonstrates that Leu-Enk and YAGFL are 850 times and 60 times more potent when nasally administered with enzyme inhibitors and

Table 1 Inhibition (%) of writhing number following nasal administration of Leu-Enk ($25\,\mu g\,kg^{-1}$) solution preparations in mice.

Sample	n	Number of writhings	Inhibition
Control	16	29.07 ± 1.14	0
L1*	6	11.80 ± 2.31	56.40 ± 8.54
L2*	6	22.20 ± 4.62	17.98 ± 7.07
L3*	8	9.13 ± 2.06	66.29 ± 4.27
L4*	11	7.82 ± 2.17	71.11 ± 8.01
Ketoprofen*	6	13.33 ± 2.06	50.73 ± 7.61

Data are expressed as the mean ± s.e. L1: Leu-Enk alone was administered 5 min before the administration of acetic acid; L2: Leu-Enk alone was administered 30 min before the administration of acetic acid; L3: Leu-Enk was administered with AM (125 μM), TM (0.5 mM), EDTA (5 mM) and LPC (0.5%) 30 min before the administration of acetic acid; L4: Leu-Enk was administered with AA (1%), TM (0.5 mM), EDTA (5 mM) and LPC (0.5%) 30 min before the administration of acetic acid. *Statistically significant ($P < 0.05$).

Table 2 Inhibition (%) of writhing number following nasal administration of YAGFL ($25\,\mu g\,kg^{-1}$) solution preparations in mice.

Sample	n	Number of writhings	Inhibition
Control	16	29.07 ± 1.14	0
Y1*	11	17.45 ± 0.77	35.50 ± 2.83
Y2*	7	11.71 ± 1.21	56.71 ± 4.47
Y3*	11	10.0 ± 1.28	63.05 ± 4.73
Y4*	7	8.0 ± 4.33	70.44 ± 11.46
Ketoprofen*	6	13.33 ± 2.06	50.73 ± 7.61

Data are expressed as the mean ± s.e. Y1: YAGFL alone was administered 30 min before the administration of acetic acid; Y2: YAGFL was administered with TM (0.5 mM) and EDTA (5 mM) 30 min before the administration of acetic acid; Y3: YAGFL was administered with TM (0.5 mM), EDTA (5 mM) and LPC (0.5%) 30 min before the administration of acetic acid; Y4: YAGFL was administered with TM (0.5 mM), EDTA (5 mM), LPC (0.5%) and PVP (5%) 30 min before the administration of acetic acid. *Statistically significant ($P < 0.05$).

absorption enhancers than literature values for ketoprofen by oral administration and morphine by subcutaneous administration, respectively (Saito & Nomura 1989).

Discussion

Although transmucosal delivery has received a great deal of attention for many compounds that are not feasible for oral delivery, it also has limitations of poor absorption and extensive hydrolysis, especially in peptides (Faraj et al 1990b). In an attempt to increase the extent of peptide absorption, enzyme inhibitors and absorption enhancers have been employed.

In the earlier permeation studies (Sayani et al 1994; Gwak et al 2002) of Leu-Enk and YAGFL using various mucosae, the highest permeation rate was seen through the nasal mucosa for both enkephalins, and this was attributed to the thickness of the mucosa and the activity of enkephalin-degrading enzymes. The nasal mucosa has been reported to have a thickness of 53.5 μm, while the thicknesses of the rectal and vaginal mucosa are 175.3 and 165.1 μm, respectively (Corbo et al 1990). In the study of enzymatic degradation of Leu-Enk and YAGFL in various rabbit mucosa extracts, the nasal mucosa was found to have a lower enkephalin-degrading enzyme activity than rectal or vaginal mucosae; the degradation half-lives of Leu-Enk were 1.62, 0.37 and 1.12 h and those of YAGFL were 30.55, 9.70 and 6.82 h, respectively (Chun & Park 1994). The mixed inhibitors that were used in this study have been shown to be effective in stabilizing enkephalins in solution (Sayani et al 1993); this could enhance the permeation of enkephalins across the nasal mucosa, possibly because of the increased stability of the enkephalins in the presence of the inhibitors, thus allowing their presence in solution long enough for permeation (Sayani et al 1994; Gwak et al 2002). It has also been reported that a direct transport pathway from nasal cavity to cerebrospinal fluid exists (Sakane et al 1994).

Based on the assumption that our enkephalin preparations are stable at the nasal cavity and nasally delivered persistently into the brain and brain circulation at least for 1 h, the writhing test was designed. As expected, L2, which was administered 30 min before the administration of acetic acid, showed a very weak analgesic effect. On the other hand, L1, which was administered 5 min before the administration of acetic acid, showed a significant analgesic effect. These results indicate that the longer duration (30 min) of non-stabilized enkephalin (L2) in the nasal cavity causes extensive degradation, and the minute amount of enkephalin delivered into the blood 40 min after nasal administration is quickly destroyed by the aminopeptidases. However, in the case of L1, since the amount of time in the nasal cavity is relatively short (15 min) before writhing, it seems that significant analgesic activity is obtained, possibly because of relatively higher nasal absorption. However, all other nasal solutions of Leu-Enk and YAGFL containing mixed enzyme inhibitors showed marked analgesic activities even up to 50 min after nasal administration.

It was suggested that aminopeptidase, enkephalinase, dipeptidase and carboxypeptidase are responsible for the degradation of enkephalins. Of these, aminopeptidase and enkephalinase have been shown to be the major enzymes, and are responsible for up to 90% of the degradation (Schwartz et al 1981). However, other studies (Chun & Park 1994; Chun et al 1996b) have demonstrated that the hydrolytic fragments of YAGFL in all mucosa extracts, including the nasal mucosa, were mainly Tyr-D-Ala-Gly and Phe-Leu-amide, indicating that this synthetic analogue was resistant to aminopeptidase. We therefore did not use the most potent aminopeptidase inhibitor, AM (Barclay & Phillips 1980), for the study of YAGFL. EDTA has been employed as the inhibitor against the enkephalinase (Malfroy et al 1978). TM, which has been used as a bacteriostatic and fungistatic in several topical pharmaceutical products, showed strong inhibition of aminopeptidases and dipeptidyl carboxypeptidase in the

earlier study of methionine enkephalin (Chun & Chien 1993). Medium-chain-length dicarboxylic acid (C_8–C_{13}) is known to be a competitive inhibitor of tyrosinase (Passi et al 1989). As a preliminary study, we investigated the effect of medium-chain-length dicarboxylic acid on the degradation of Leu-Enk in the nasal, rectal and vaginal extracts of rabbit according to the method described by Chun & Park (1994). Three acids of AA (C_9), sodium laurate (LA; C_{12}) and 1,12-dodecanedioic acid (DA; C_{12}) at a concentration of 1% were compared. AA was found to be the most effective enzyme inhibitor of Leu-Enk in the nasal extract while LA and DA showed high inhibition in the rectal and vaginal extracts, respectively.

Previously, the effect of several absorption enhancers on the nasal absorption of recombinant human growth hormone (hGH) has been examined (O'Hagan et al 1990; Fisher et al 1991). In these studies, it was demonstrated that LPC was an effective enhancer of the nasal absorption of hGH in rats, increasing the relative bioavailability to about 26%. In other studies, LPC was also shown to be effective in increasing the bioavailability of nasally applied gentamicin (Illum et al 1988) and insulin (Illum et al 1989; Farraj et al 1990). LPC is known to have some special advantages as an enhancing agent (Fisher et al 1991). It is a surface-active amphiphile, which exists in most biological membranes at low concentration. It acts as a penetration enhancer at low concentration and is converted to normal cell components.

Povidone was used for increasing the viscosity of the solution preparation in this study. Its enhancing effect was possibly caused by the longer duration of the peptide in the nasal cavity.

In our previous in-vitro study (Gwak et al 2002), it was found that Leu-Enk itself did not permeate through the nasal mucosa over 24 h, and the addition of inhibitors markedly enhanced permeation; the flux of Leu-Enk increased from 0 to 20.7 $\mu g\,cm^{-2}\,h^{-1}$. YAGFL alone, however, had considerable flux through the nasal mucosa (4.36 $\mu g\,cm^{-2}\,h^{-1}$), and the addition of inhibitors failed to show a marked increase in flux (4.78 $\mu g\,cm^{-2}\,h^{-1}$). The further addition of LPC enhanced the permeation flux of Leu-Enk and YAGFL to 25.9 and 21.4 $\mu g\,cm^{-2}\,h^{-1}$, respectively. In this in-vivo experiment, YAGFL itself (Y1) had a high inhibition rate of writhing compared to Leu-Enk (L2), which was consistent with the in-vitro results. The addition of inhibitors and permeation enhancers increased the analgesic effect of both enkephalins dramatically, to a value higher than that of ketoprofen.

We employed ketoprofen as a reference compound because of the easy access and relatively high stability compared to narcotic compounds or subcutaneous administration of enkephalins. The ED50 value of orally administered ketoprofen in mice was reported to be 11.3 $mg\,kg^{-1}$ in the literature (Amanuma et al 1984). This value is similar to our result, showing that the inhibition rate was 50.7% when ketoprofen was administered orally at a dose of 10 $mg\,kg^{-1}$.

Although the number of animals that responded (6–16) is not sufficient in the writhing study, the results obtained give some information on analgesic activity depending on the nasal preparation.

Conclusions

In conclusion, the results of this study show that Leu-Enk or YAGFL could exert very high analgesic activity when nasally administered with mixed inhibitors and absorption enhancers as compared to other analgesics. Nasal delivery of enzymatically stabilized enkephalin formulations is therefore a very promising means of maximizing the pharmacological activities of both natural and synthetic enkephalins and for prolonging their activities.

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